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THE ACTION OF TRIETHYLENEMELAMINE ON THE FERTILITY OF MALE RATS

BY

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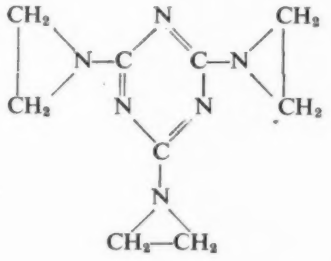
From the Christie Hospital and Holt Radium Institute, Manchester

(RECEIVED APRIL 21, 1956)

Small doses of triethylenemelamine (TEM) had a selective action on the fertility of male rats. One dose (0.2 mg./kg., i.p.) produced effects ranging from subfertility to sterility during the next 3 weeks. In the fourth week sterility was the rule, but normal fertility was restored in the fifth week. A short course of the drug (5 daily doses, 0.2 mg./kg., i.p.) resulted in sterility lasting about 5 weeks, after which fertility was rapidly regained. Daily doses (0.05 mg./kg., i.p.) caused infertility in about a week which was maintained throughout treatment (7 weeks), and persisted for several weeks after the drug was discontinued. Sexual activity of infertile animals seemed normal and sperm production appeared to continue. Spermatozoa from infertile animals were able to reach and penetrate ova. The results suggest that TEM acts directly on the germinal epithelium. An attempt has been made to provide some explanation for these results and correlate them with the time required for spermatogenesis.

Recent studies of the inhibitory effects of triethylenemelamine (TEM) on the growth of the Walker tumour involved the prolonged administration of this substance to rats (Jackson, 1954). During these experiments tumour-bearing animals of both sexes were kept together, and it was noticed that no litters were produced. It seemed of interest to investigate this effect, with the result that a selective action of triethylenemelamine on the fertility of male rats has been found. In the female rat, the drug prevents the development of the early embryo. A brief account of some earlier results has been published (Jackson and Bock, 1955).



Triethylenemelamine (TEM)

MATERIALS AND METHODS

Triethylenemelamine, a trifunctional ethyleneimine, was prepared according to the method described by Bestian (1950). Both the crystalline compound and its aqueous solution were stored at 5° C. In view of its reputed instability, solutions of the drug were renewed at intervals of about one week as a precautionary measure.

The rats used were an inbred American Wistar strain maintained on a cube diet supplied by the North-Eastern Agricultural Society. In earlier experiments, the fertility of male rats was assessed by group mating—for example, 5 males and 10 females were allowed to remain together in one cage for a period of 14 days, after which the females were removed and housed in separate boxes ; such a technique is only of value when the dose of drug given is sufficient to render all the males infertile. Its limitations led to the use of isolated mating, in which paired animals were housed in separate boxes and the course of events followed by examination of vaginal smears as early as possible each morning. Successful impregnation was almost always associated with the detection of spermatozoa in the vaginal smear ; very occasionally sperm were not seen but litters were produced. Insemination usually occurred within 6 days of pairing, often in the first 4 days. The final routine adopted was to pair off treated males with females and change the latter each week. Insemination by treated, infertile males still producing spermatozoa was followed by pseudopregnancy lasting about 15 days, during which time there was no evidence of further impregnation. As far as possible the female rats used were of established fertility, but on occasions recourse had to be made to virgin animals. For some purposes females in oestrus were selected in order to enhance the likelihood of successful mating on a particular occasion. Control groups of either sex were strictly comparable with those of experimental groups.

Results

Table I shows the combined results of two similar experiments in which persistent treatment of male rats with TEM was carried out (42 doses in 57 days, at the rate of 0.05 mg./kg. each).

TABLE I

EFFECT OF REPEATED ADMINISTRATION OF TRIETHYLENEMELAMINE ON THE FERTILITY OF MALE RATS (42 DOSES, 0·05 MG./KG., I.P., IN 57 DAYS)

Each of 5 treated male rats were paired at various times with females of established fertility. During each period, insemination was detected within the time range indicated in the second column. The table is a composite one derived from two similar experiments.

Mating Period	Time Range of Inseminations (Days from 1st Dose Incl.)	No. of Females Found Inseminated	No. Producing Litters	Individual Litter Size
1 *	9–11	5/5	1/5	0, 0, 0, 3, 0
2	15–17	4/5	0/5	0
3	35–37	4/5	0/5	0
	TREATMENT DISCONTINUED ON DAY 57			
4	62–63	3/5	0/5	0
5	74–75	3/5	0/5	0
6	87–90	4/5	0/5	0
7	92–94	5/5	3/5	0, 5, 3, 0*,
8	107–108	5/5	4/5	10, 5, 11, 0, 8
9	109	5/5	4/5	0, 12, 7, 6, 12

* Unknown number of young eaten.

Sterility was soon induced and was maintained not only during treatment but persisted for about 30 days after the last dose. Thus inseminations occurring between 87 and 90 days from the commencement of treatment were unproductive, whilst those taking place between 92 and 94 days indicated that fertility was returning. By the 109th day 8/10 (8 of 10) animals showed normal fertility. Throughout the period of sterility, both during and after treatment, sperm production apparently continued as indicated by their presence in vaginal smears. The sperm content of vaginal smears was similar in females mated with either treated or untreated males. No change in the sexual behaviour of the treated animals was observed.

The administration of one dose of TEM (0.2 mg./kg., i.p.) produced complex effects (Table II). Treated males were mated after a lapse of 24 hr. to allow clearance of the drug, when apparently normal insemination occurred in 9/10 pairs between the third and sixth day according to the oestrous cycles of the females concerned. Only 5/10 produced litters compared with 5/5 in the corresponding control series, although the actual litter sizes were comparable in each group (Table II, week 1). In a subsequent experiment, again using 10 treated males, insemination occurred in 9/10 females between the second and fourth day, but only three litters were produced. In neither of these experiments was there any relation between time after the dose when insemination occurred and the successful production of litters. Referring again to the experiment shown in Table II, 8/10 females were impregnated between day 8 and 10 after the dose of TEM (week 2). Of these, 7 produced litters, but the number of offspring in each was much reduced compared with the controls; this subfertile state also extended into the third week of the experiment. It was noteworthy that during these three weeks of mating sterility was not a constant feature in any one male. A striking change occurred in the fourth week of the experi-

TABLE II

EFFECT OF ONE DOSE OF TRIETHYLENEMELAMINE (0·2 MG./KG., I.P.) ON THE FERTILITY OF MALE RATS

Each vertical column shows the results of pairing 10 treated male rats for six consecutive weeks with females of established fertility. They are to be compared with those from 5 untreated males in a concurrent experiment. Each rat was numbered and the litter data are to be read horizontally at the appropriate level. Weekly fluctuations in fertility are pronounced in each treated male during the first 3 weeks, with uniform sterility in the 4th and restoration of normal fertility in the matings of the 5th and 6th weeks.

	TREATED						CONTROLS					
Week	1	2	3	4	5	6	1	2	3	4	5	6
Time range of inseminations (days after dose)	3–6	8–10	Not examined	23–26	30–33	36–39	3–6	8–11	Not examined	23–25	30–33	36–38
No. of females found inseminated	9/10	8/10	—	9/10	9/10	9/10	4/5	4/5	—	5/5	5/5	5/5
No. of litters produced	6/10	7/10	8/10	1/10	9/10	8/10	5/5	4/5	4/5	5/5	5/5	5/5
Individual litter size:												
Rat No. 1	0	4	1	0	8	9	9	4	11	3	9	9
2	0	0	2	0	0	0	9	0	12	12	6	9
3	5	0	5	0	6	11	8	11	13	11	12	12
4	8	3	3	0	7	0	5	13	0	11	9	9
5	0	0	5	0	7	4	8	10	13	4	9	9
6	8	0	3	0	10	8						
7	†	3	6	0	10	3						
8	9	5	5	1 (d)	5	9						
9	0	3	0	0	6	7						
10	5	2	0	0	5	12						

† = Unknown number of young eaten. (d) = Dead foetus.

TABLE III

FREQUENCY OF MATING OF MALE RATS IN RELATION TO THE ACTION OF TEM (ONE DOSE, 0·2 MG./KG., I.P.)

In matings before the drug was administered the male remained each night with 5 females in oestrus, and in the post-treatment matings two females in oestrus/male were used on each occasion. The females used were of established fertility, and all the positive vaginal smears showed masses of spermatozoa. In spite of repeated matings the changes in fertility followed a similar sequence as when single weekly matings were used (Table II).

		Drug Given ↓						POST-TREATMENT									
	Days	4	3	2	1	0	2	4	5	7	8	11	15	20	22	24	28	34	42
MALE A	No. of females inseminated	1	2	0	1		0	1	1	0	2	1	1	1		0	1	1	1
	No. producing litters	1	2	0	1		0	1	0	0	0	1	1	0		0	1	1	1
	Litter size	6	4, 5	0	11		0	6	0	0	0	4	1	0		0	6	9	13
MALE B	No. of females inseminated	1	0	2	2		1	1	0	0	2	1	1	0	1	1	1	1	1
	No. producing litters	1	0	1	1		1	1	0	0	0	0	0	0	0	0	1	1	1
	Litter size	4	0	10	6		4	2	0	0	0	0	0	0	0	0	8	8	12

ment, for, in spite of "normal" insemination of 9/10 females, between the 23rd and 26th day one animal only gave birth to a dead foetus. None of the others produced litters or, indeed, showed any signs of pregnancy. However, impregnation of 9/10 females during the 5th week (between days 30 and 33) resulted in normal-size litters from each animal, and a similar result was obtained from the final period of mating (week 6). Comparable results have emerged from other experiments using the same dose of the drug. In these experiments, each female remained with the male for a week, insemination occurring at oestrus when ovulation takes place spontaneously. The manifest effects of TEM on fertility might depend on the frequency of mating with the removal of available mature spermatozoa, as well as on the rate of replenishment. Table III records the changes in fertility of two male rats paired frequently with females in oestrus after the injection of one dose of the drug (0.2 mg./kg.). For several nights before the drug was given, each male was mated with groups of five females in oestrus in an attempt to deplete any sperm reservoir, but surprisingly few were inseminated. After the drug, matings were continued, using 2 new females per male at the times shown. Inseminations continued at a similar rate as in the pretreatment period, and the changes in fertility were comparable to those observed when one mating each week was the routine (Table II). The sperm content of vaginal smears was similar in pretreatment and post-treatment matings.

The effect on fertility of five daily doses of TEM (0.2 mg./kg., i.p.) has been examined on a number of occasions. The combined results of two experiments are shown in Table IV. Such a course of treatment effectively inhibits the growth of certain rat tumours (Walker carcinosarcoma, Jensen sarcoma) which accounts for its application in the present work. Before commencing the series of matings, it was thought prudent to allow at least 48 hr. to elapse after the last dose of TEM to ensure adequate clearance of the drug. Sterility of the males was the rule from the eighth day after the first dose, and this condition persisted for 25 to 29 days after the final dose. Fertility had returned in 2/5 animals 33 days from the last dose and recovery was complete 38 to 40 days from the end of treatment. These figures are

TABLE IV

EFFECT OF TRIETHYLENEMELAMINE (5 DAILY DOSES, 0·2 MG./KG., I.P.) ON THE FERTILITY OF MALE RATS

Results of post-treatment pairings of 5 treated males with females of established fertility. Note the prolonged infertility associated with seemingly normal inseminations. The restoration of normal fertility ultimately occurs quite rapidly.

Mating Period	Time Range of Inseminations (Days from 1st Dose Incl.)	No. of Rats Inseminated	No. Producing Litters	Individual Litter Size
1	8–12	5/5	0/5	0
2	12–17	5/5	0/5	0
3	20–24	5/5	0/5	0
4	30–34	5/5	0/5	0
5	37–42	5/5	2/5	0, 0, 5, 3, 0
6	43–45	5/5	5/5	4, 6, 10, 4, 7
7	52–53	5/5	4/5	9, 0, 6, 10, 8

based on the known dates of insemination by individual males. Throughout these experiments the sperm content of vaginal smears was similar in both control and treated groups of animals. Repeated injections of TEM (0.2 mg./kg.) distributed over a prolonged period of time (21 doses in 57 days) produced infertility in a group of rats which was maintained long after the drug had been discontinued (Jackson and Bock, 1955). Even 75 days after the last dose, fertility had returned in only one animal. Spermatozoa were found in

vaginal smears from 4/5 females 15 to 20 days after the last dose, but their numbers were obviously reduced compared with the corresponding controls. In subsequent matings sperm were encountered only sporadically. This treatment produced obvious toxic effects such as failure to gain weight and general lack of well-being.

It has been shown that spermatozoa produced by rats rendered infertile with TEM can reach and penetrate ova, although no evidence of embryonic development was seen in the uterine horns of females examined at intervals up to 14 days after insemination. For example, five treated male rats (after 25 doses of TEM, 0.05 mg./kg. in 30 days) were mated separately with females in oestrus. The next morning (12 hr. later) vaginal smears indicated that insemination of four had occurred, so these animals were killed and shed ova removed from the Fallopian tubes. From each animal ova were recovered into which sperm had penetrated. In all 11/25 ova contained spermatozoa. Since the male rats used had previously been shown to be infertile, they were re-mated 24 hr. after the above experiment. In spite of apparently normal inseminations during the next few days no litters were produced (Table I, period 3). The initial fertility of these males is established by the recovery of fertility some weeks after treatment was discontinued. In a comparable experiment with untreated males, 38 ova were recovered, 33 of which contained spermatozoa. Similar results were obtained during the infertile period after five daily doses of TEM (0.2 mg./kg., i.p.). Five treated males were paired with females in oestrus on the 28th night after the first dose. By the next morning two animals had been inseminated and were killed about 12 hr. after mating. Shed ova were recovered with the following results:

Female 1. R tube: Four ova found, all containing sperm. In two ova the nuclei had fused; in the others the sperm were lying free in the cytoplasm. L. tube: Two ova found, both containing sperm.

Female 2. R. tube: Five ova seen, four containing sperm. L. tube: Four ova found, one containing a spermatozoon.

The two females killed were replaced and the five paired animals then constituted the group referred to in Table IV (mating period 4), from which no litters resulted. All the males of the group later recovered their fertility.

When litters were born after mating with treated males, the period of gestation was within normal limits and no superficial abnormalities were seen in the offspring, which were usually kept for three weeks.

Discussion

Triethylenemelamine is a highly toxic substance, its pharmacological effects being generally ascribed to its ability to react chemically with undetermined but vital cell components and thus interfere with the process of cell division. The results may be manifest, for example, as toxic effects on the susceptible bone marrow and alimentary tract or as inhibition of the growth of some varieties of experimental tumour. On the other hand, mutagenic changes may be induced or neoplastic transformations brought about (Hendry, Homer, Rose, and Walpole, 1951; Walpole, Roberts, Rose, Hendry, and Homer, 1954). The great susceptibility of the human subject to the action of TEM is well recognized (Haddow, 1953; Nabarro, 1953) although rodents are more tolerant, the mouse more so than the rat. It may be that these differences are related to the ability of the recipient species to metabolize the drug, and that too much emphasis has been laid previously on its chemical reactivity (Craig and Jackson, 1955).

In view of the rapid rate of cell division in the testis, the susceptibility of this organ to damage by TEM is not unexpected and is, at first sight, in keeping with the well-known radiomimetic properties of the drug. The histological picture of destruction in the testis of the dog and the rat after TEM was noted by Hendry *et al.* (1951) during an extensive survey of the tumour-inhibiting properties of ethyleneimines. They observed that a lethal dose of TEM to rats produced effects on the germinal epithelium ranging from inhibition of spermatogenesis to complete atrophy. The present work indicates that TEM can produce more subtle effects on spermatogenesis than is suggested by the picture of inhibition and destruction referred to above. Evidence has been presented which shows that the entire sequence of sperm production may be affected, possibly by interference at certain key positions. Spermatogenesis is a process of great complexity (Fig. 1) and little is known about the factors which initiate and regulate its many stages. However, a working hypothesis has been developed which provides some explanation for the effects produced by TEM. It has been necessary to assume that spermatogenesis goes on continually and that, over the testis as a whole at any given time, constant proportions of the germinal epithelium are occupied in particular phases of the process. In this connexion, it has been reported that mitotic activity in the testis of the mouse and rat is constant throughout the day (Bullough, 1948; Clermont and Leblond, 1953), whilst close agree-

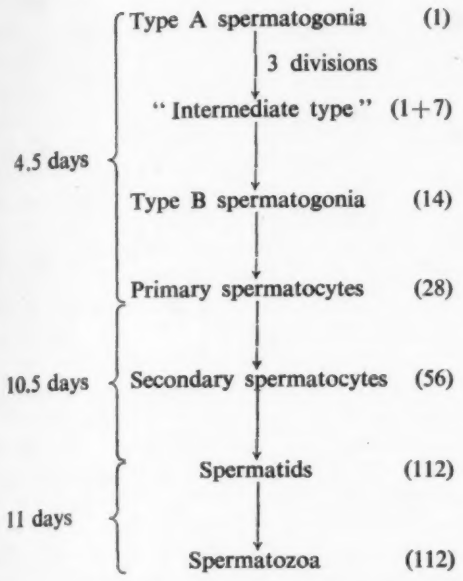


FIG. 1.—Outline of the development of spermatozoa. (Adapted from work by Leblond and Clermont (1952) and Oakberg (1955).) The estimated number of cells originating from one spermatogonium is shown in brackets for each stage. The population of spermatogonia is said to be maintained by one out of the 8 cells arising from three initial divisions of a type A spermatogonium. The duration of the stages (Leblond and Clermont, 1952) is based on an estimate of 26 days as the time required for spermatogenesis.

ment in % of tubules present in any given stage of the seminiferous cycle has been observed in different rats (Leblond and Clermont, 1952).

The most notable feature which follows a single injection of TEM is a period of sterility associated with inseminations occurring between 23 and 26 days after the dose (Table II). This suggests a well-defined action of the drug on an early stage of the spermatogenic process, its specific nature being emphasized by the production of normal-size litters after the next inseminations between 30 and 33 days. It follows that generations of cells succeeding those affected by the drug are unharmed and implies that waves of spermatogenic activity passing along the many seminiferous tubules are co-ordinated and in phase. The rapid restoration of normal fertility also indicates that the available mature sperm was used at each impregnation. Other changes in fertility after one dose of TEM show that later stages in spermatogenesis are affected. The marked reduction in litter size following matings of the second and third weeks (Table II) appears to be best explained by inseminations with mixtures of normal and infertile spermatozoa. Since the latter have been shown to be able to reach and penetrate ova, the subfertility could be due to competition for available ova by the two kinds of sperm. The normal sperm may originate from a closely associated phase of spermatogenesis which is less susceptible to the drug. This is not unreasonable, since the development of spermatozoa involves many stages and morphological changes. In fact, the results of a short exposure to the compound may well correlate with the time required to complete various stages of spermatogenesis. It is difficult to understand why so many inseminations occurring during the first six days after the drug were non-productive, whilst others resulted in litters of normal size. This action of the drug is presumably associated with late stages of spermatogenesis or even with mature sperm, but there is no apparent relation between the sterility of the mating and the time after the dose when impregnation occurred. An antimitotic effect is excluded since the formation of mature sperm from spermatids requires 11 days and does not involve cell division. Another unexplained result is the significant but irregular variation from fertility to sterility occurring in individual males from week to week (Table II).

The question of the influence of frequency of effective mating in experiments of this nature is an important one. How far any existing store of mature sperm in the rat is utilized during insemination is, so far as we are aware, unknown. From experiments carried out during the present investigations, it appears that the rat rarely inseminates more than one or two females in oestrus per night ; also that periods of one or two nights recur during which there is no evidence of insemination. It is interesting that the overall effects on fertility of one dose of TEM seem to be independent of previous copulations or the frequency of mating after the treatment. Physiological considerations appear to govern the results, so that insemination of one female each week (Table II) reflects the changes induced in the spermatogenic process as effectively as more frequent matings (Table III). This matter is being examined in more detail.

Shaver (1953) has estimated that, in the rat, about 27 days are required for mature sperm to be formed from spermatogonia. This figure was derived from a histological study of the effect of whole-body radiation on the testis, which caused complete inhibition of mitosis in spermatogonia (and possibly in more primitive cells) lasting for about four weeks. Later stages were apparently unaffected by the treatment, so that a maturation depletion of the germinal epithelium occurred, which was complete in 27 days. Thereafter a re-

population of the epithelium occurred, requiring 4 to 5 months for completion. The parallel between Shaver's estimate of 27 days and the occurrence of an infertile period 23 to 26 days after one dose of TEM is very striking. In the drug experiment, however, it is evident that only sterile sperm were available for copulations between 23 and 26 days after the dose and that these were rapidly replaced by fertile ones; thus, without an intermediate mating, full fertility was present at the latest 30 to 33 days after the injection. By analogy with Shaver's work, it appears that a comparable early stage in spermatogenesis is most susceptible to TEM. The available evidence suggests that small doses of TEM impose modifications on the sperm-producing mechanism so that normal-looking but infertile sperm are produced. There is no indication of a maturation depletion such as Shaver describes after irradiation, for the speedy restoration of normal fertility could not then occur.

Short courses of TEM (Table IV) produce more drastic effects on fertility. All animals were sterilized for 3 to 4 weeks after the last dose, but during this period inseminations continued at seemingly normal levels. Ova can be reached and penetrated by these sterile sperm, but, owing to some change due to the drug, embryonic development does not proceed. In this connexion it is known that spermatozoa of *x*-irradiated rats can retain motility and fertilizing power, but are unable to initiate normal development of the ovum (Brennecke, 1937). The return of fertility after several doses of TEM is also quite rapid; from the dates of insemination of individual animals fertility had returned 35 to 42 days after the last dose, no remarkable prolongation over the re-establishment of normal fertility after one dose of TEM. The development of sterility in about a week with small daily doses of TEM (Table I) emphasizes the overall sensitivity of spermatogenesis to the drug and shows that the effect is cumulative. In these prolonged experiments, sterility is associated with continued sperm production as evidenced by insemination findings and the sperm complement of the cauda epididymis from animals killed during and after the course of treatment. Frequent administration of the drug should ensure that successive generations of cells passing through susceptible phases of spermatogenesis become modified. Once the entire sequence has been affected discontinuation of treatment must be followed by persistence of sterility. The time required for fertility to return should be a measure of that needed for the generation of spermatozoa from the earliest site of action of the drug. After two months of infertility during treatment (Table I), fertility had returned in 2/5 animals 35 days after the last dose. Unfortunately more exact data are not available owing to the timing of the mating periods. It seems clear, however, that more intense or prolonged treatment in these various experiments does not unduly retard the recovery process and points to a common focus of action on an early stage of spermatogenesis. It is hoped that further experiments now in progress combined with histological studies will give more precise information and help to locate the sensitive phases. The possible rôle of oligospermia caused by the drug has also to be considered, but an accurate evaluation of this can only be made by sperm counts—a difficult problem in these small animals. However, the normal sperm content of the vagina after insemination by treated sterile animals and the ability of these sperm to reach and penetrate ova suggest that a reduced sperm output is not a major factor.

Although TEM is a highly toxic substance, the lower doses used produced little evidence of untoward effects apart from some interference with gain of weight. Blood examinations were not carried out, but Crossley, Allison, Wainio, and Muenzen (1951) found a similar dose rate (0.05 mg./kg. daily) caused a mild leucopenia in their rats; half this dose produced neither leucopenia nor other demonstrable toxic effect. Walpole *et al.* (1954) have shown that injections of TEM in arachis oil are actively carcinogenic, but no tumours were obtained after the administration of aqueous solutions. In the present work only aqueous solutions have been used and treated rats have not shown any noticeable tendency to develop tumours.

The relation between chemical structure and antifertility action is being investigated. So far, some other ethyleneimines have been shown to produce similar effects to triethylenemelamine, but comparable activity has not been observed among a wider range of antimitotics examined, including other radiomimetic compounds of the alkylating type.

The authors are much indebted to S. Muldal, Cand. real., for demonstrating that sterile sperm from TEM-treated male rats were able to reach and penetrate ova.

References

Bestian, H. (1950). *Liebigs Ann.*, **566**, 210.
Brennecke, H. (1937). *Strahlentherapie*, **60**, 214.
Bullough, W. S. (1948). *Proc. roy. Soc. B.*, **135**, 212.

Clermont, Y., and Leblond, C. P. (1953). *Amer. J. Anat.*, **93**, 475.

Craig, A. W., and Jackson, H. (1955). *Brit. J. Pharmacol.*, **10**, 321.

Crossley, M. L., Allison, J. B., Wainio, W. W., and Muenzen, J. B. (1951). *J. Nat. Cancer Inst.*, **12**, 305.

Haddow, A. (1953). *Proc. R. Soc. Med.*, **46**, 692.

Hendry, J. A., Homer, R. F., Rose, F. L., and Walpole, A. L. (1951). *Brit. J. Pharmacol.*, **6**, 357.

Jackson, H. (1954). *Brit. J. Cancer*, **8**, 336.

—— and Bock, M. (1955). *Nature (Lond.)*, **175**, 1037.

Leblond, C. P., and Clermont, Y. (1952). *Amer. J. Anat.*, **90**, 167.

Nabarro, J. D. N. (1953). *Proc. R. Soc. Med.*, **46**, 696.

Oakberg, E. F. (1955). *Radiation Res.*, **2**, 372.

Shaver, S. L. (1953). *Amer. J. Anat.*, **92**, 391.

Walpole, A. L., Roberts, D. C., Rose, F. L., Hendry, J. A., and Homer, R. F. (1954). *Brit. J. Pharmacol.*, **9**, 306.

Brit. J. Pharmacol. (1957), **12**, 8.

EFFECTS OF RESERPINE ON BLOOD-PRESSURE RESPONSES EVOKED FROM THE HYPOTHALAMUS

BY

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Different areas of the hypothalamus were stimulated by a stereotaxic technique before and after reserpine in unanaesthetized cats. Reserpine in a dose of 1 mg./kg. body weight increased the threshold of stimulation of hypothalamic areas in cats giving an initial pressor response. It decreased the threshold of stimulation of areas of the hypothalamus in animals giving an initial depressor response. Reserpine thus appeared to affect the hypothalamus directly in two ways. It depressed the sympathetic centres in the diencephalon and facilitated the parasympathetic ones.

Experimental pharmacological investigations by various workers (Bein, 1953; Tripod and Meier, 1954; Barrett, Rutledge, and Rogie, 1954) have revealed that in therapeutic doses reserpine does not possess any significant peripheral actions on the cardiovascular system. The ability of the drug to block certain reflex vasopressor responses (those obtained from carotid occlusion, stimulation of the central end of the cut vagus or of the sciatic nerve (Bein, 1953; Ray, Roy, Dasgupta, and Werner, 1953; Trapold, Plummer, and Yonkman, 1954), together with its clinical effects (hypotension, bradycardia, miosis, and tranquillizing action), certainly point to a central action, but they do not offer unequivocal support for the hypothesis of a hypothalamic site. Bhargava and Borison (1955) point out that reserpine mainly depresses the supraspinal structures without significantly affecting the spinal vasomotor excitatory state. Schneider (1955) concluded that reserpine caused a central block or inhibition of afferent impulses which normally stimulate the sympathetic activity rather than a direct depression of diencephalic sympathetic centres. Harrison and Goth (1955) observed that hypothalamic pressor response can be diminished in cats by reserpine. All the previous studies have been done on anaesthetized animals. Administration of an anaesthetic in itself may interfere with the nervous responses. The present investigation was therefore undertaken to study the effects of reserpine on hypothalamic stimulation responses in unanaesthetized animals by permanent implantation of electrodes, and to find out its effect on the hypothalamus.

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METHODS

In 22 experiments in cats, electrodes were permanently implanted on both sides of the hypothalamus at different co-ordinates with the help of the Horsley-Clarke stereotaxic instrument according to the method described by Delgado and Anand (1953). The cats were allowed to recover completely. After a period varying from 10 to 15 days, each cat was prepared for carotid blood-pressure recording under light ether anaesthesia, and allowed to regain consciousness fully. Subsequently bipolar stimulation of the hypothalamic region was done in unanaesthetized animals through the implanted electrodes by a square-wave stimulator and blood-pressure responses were recorded on a kymograph. In general, square-wave pulses of frequency of 30/sec. and 0.2 msec. duration were used. Responses were recorded with different intensities of stimulation, differing from one another by 0.5–1 volt. Reserpine ("Serpasil," Ciba), 1 mg./kg. body weight, was then injected into the femoral vein. This produced a generalized fall in blood pressure after about 2 to 3 hours, at the end of which time the same hypothalamic areas were again stimulated with the same parameters as were used before giving reserpine. Blood-pressure responses were again recorded. At the termination of the experiment the animals were killed, the brain specimens fixed in formalin, and the electrode positions verified histologically.

Results

In all 22 experiments were performed. In 16 of these stimulation of different areas of the hypothalamus before reserpine resulted in pressor responses (Table I), whereas in 6 others there were depressor responses (Table II). In 15 experiments out of 16 giving an initial pressor response, the threshold of stimulation for producing the pressor response was markedly increased after reserpine (Fig. 1 and Table I). In one experiment (No. 7, Table I, and Fig. 2), however, the threshold of stimulation for producing a pressor response was decreased after reserpine. In this cat, curiously enough, there was a general rise of blood pressure (30 mm. Hg) during the 1st and 2nd hours after giving reserpine, whereas in all others there was a general fall of blood pressure (30 to 70 mm. Hg) 2 to 3 hours after reserpine. In 6 animals

TABLE I

COMPARISON BETWEEN THE THRESHOLD INTENSITIES OF STIMULATION OF THE HYPOTHALAMIC REGIONS FOR PRODUCING A PRESSOR RESPONSE BEFORE AND AFTER RESERPINE

Serial No.	Threshold Intensity, in Volts, for Pressor Response	
	Before Reserpine	After Reserpine
1	7·7	10·0
2	10·9	14·3
3	12·3	14·3
4	4·9	10·0
5	4·9	5·4
6	4·3	12·2
7	4·35	2·55
		After reserpine the general blood pressure was raised
8	2·55	2·55
9	2·55	7·5
10	5·3	7·5
11	4·3	5·3
12	5·3	6·3
13	2·55	3·4
14	1·8	3·4
15	2·55	6·3
16	2·55	No rise even with 7·5 V.

TABLE II

COMPARISON BETWEEN THE THRESHOLD INTENSITIES OF STIMULATION OF THE HYPOTHALAMIC REGIONS FOR PRODUCING A DEPRESSOR RESPONSE BEFORE AND AFTER RESERPINE

Serial No.	Threshold Intensity of Stimulation, in Volts, for Depressor Response	
	Before Reserpine	After Reserpine
1	7·7	3·5
2	7·7	3·4
3	10·0	5·4
4	10·0	3·4
5	3·4	0·83
6	5·3	4·35

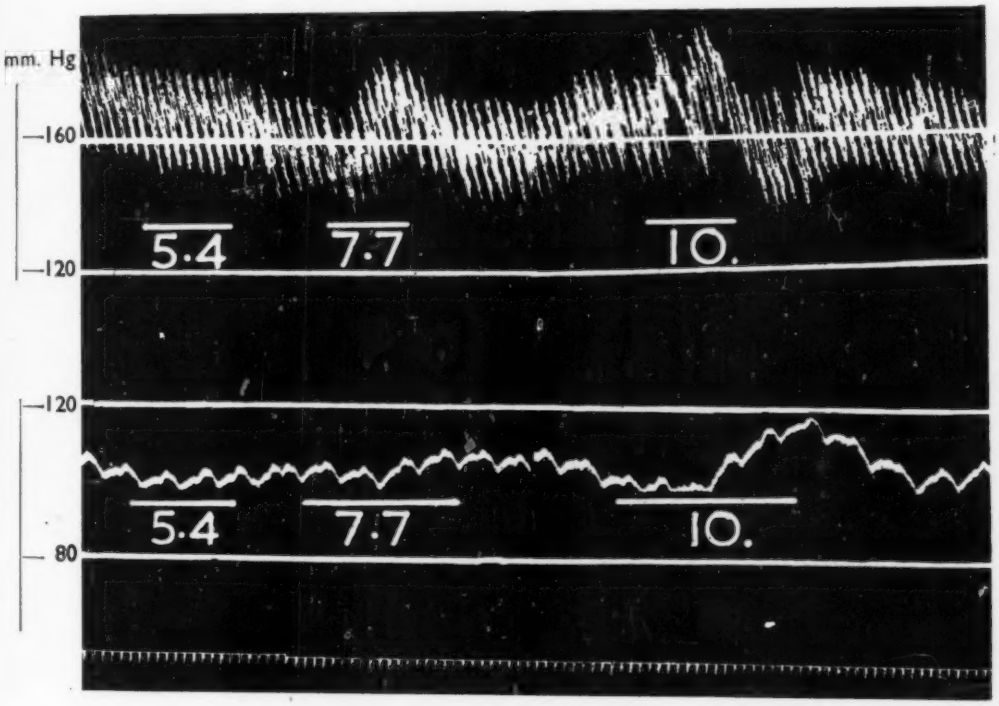

FIG. 1.—Blood-pressure responses produced by stimulation of hypothalamus, before (upper curve) and 2¼ hr. after giving reserpine (lower curve). Note the increase in the threshold of stimulation for pressor response from 7.7 V. to 10 V. after reserpine. Time signal 30/min. The horizontal line denotes the duration and the numerals the voltage of the stimulation.

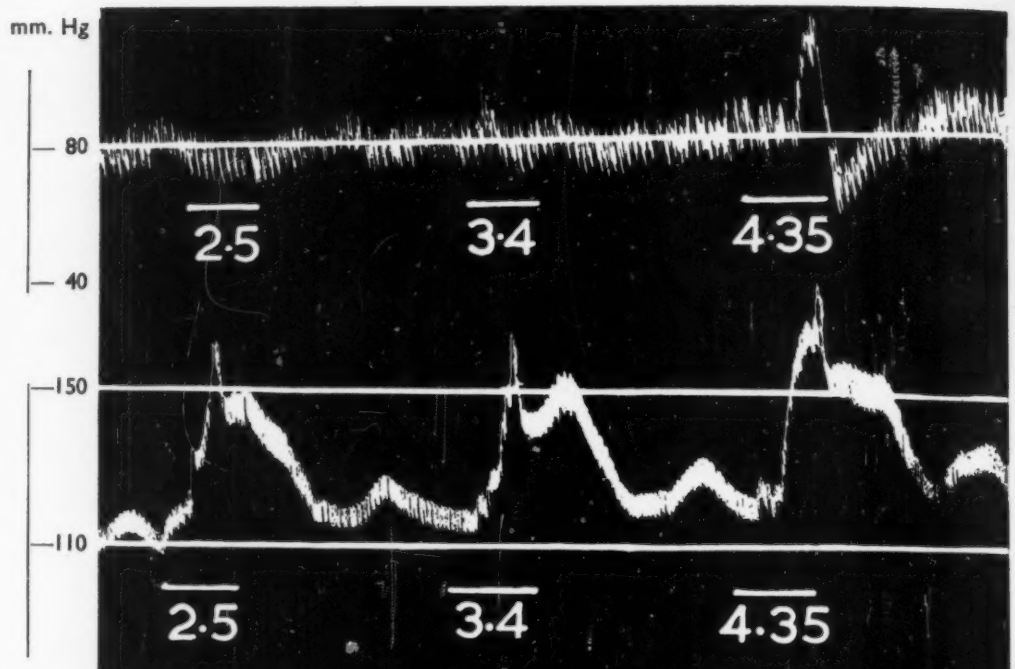

FIG. 2

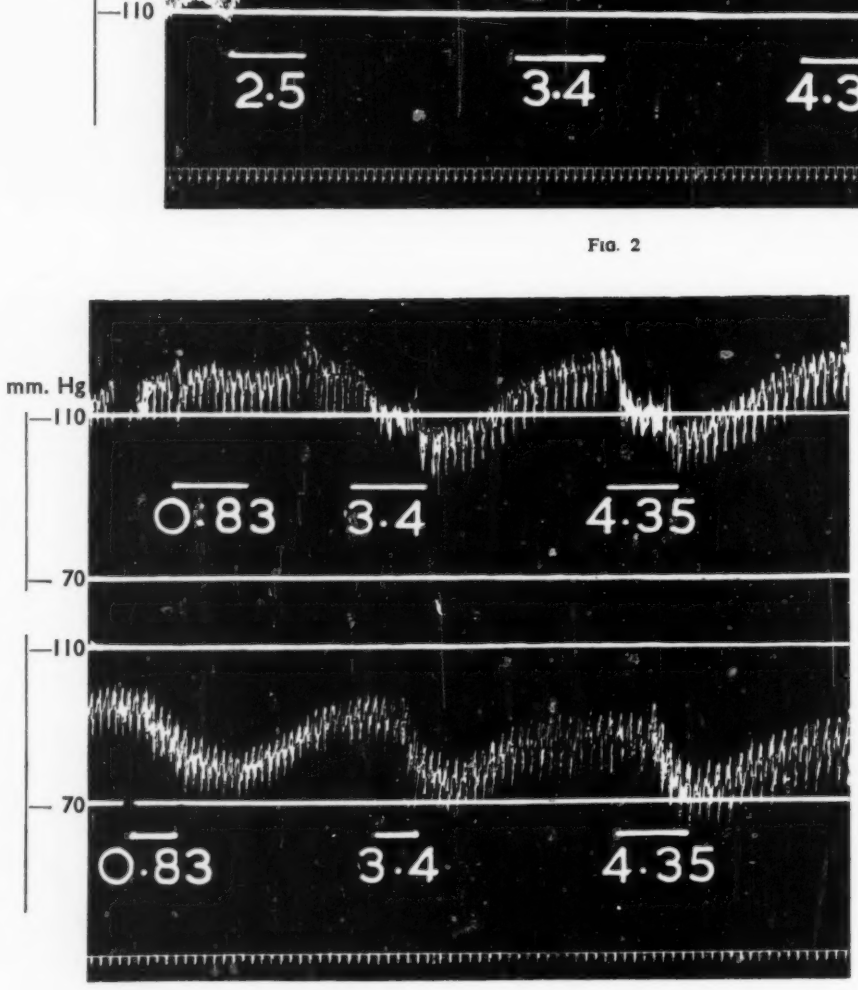

FIG. 3

FIG. 2.—Blood-pressure responses as in Fig. 1 produced by stimulation of hypothalamus, before and 4 hr. after giving reserpine, showing a decrease in the threshold for stimulation for pressor response from 4.35 V. to 2.5 V. after reserpine. Note also a rise of 30 mm. Hg in the general blood pressure after giving reserpine. Time signal 30/min.

FIG. 3.—Blood-pressure responses as in Fig. 1 produced by stimulation of hypothalamus, before and 2½ hr. after reserpine, showing a decrease in the threshold for stimulation for depressor response from 3.4 V. to 0.83 V. after reserpine. Time signal 30/min.

showing an initial depressor response, there was a marked diminution in the threshold of stimulation for producing the depressor response after reserpine (Fig. 3 and Table II). Two other cats died 3 to 4 hours after giving reserpine, owing to a continuing fall in the general blood pressure.

Discussion

The observations reported here show that after reserpine the threshold of stimulation of the hypothalamic regions giving an initial pressor response is increased. Only in one cat was the threshold of stimulation diminished. In this cat, paradoxically, there was a general rise of blood pressure after reserpine. We have been unable to find any explanation for this anomaly. On the other hand, the threshold of stimulation of those regions which gave an initial depressor response was lowered after reserpine. These observations show that reserpine, in doses of 1 mg./kg. body weight, selectively affects the hypothalamus. In such doses it not only depresses the sympathetic centres in the hypothalamus, but it also facilitates the parasympathetic centres. These results are at variance with those of Schneider (1955) and of Harrison and Goth (1955). This may be due to the fact that these authors experimented with anaesthetized animals. The anaesthetic in itself may depress the hypothalamic areas so much that it may interfere with the nervous responses evoked from the hypothalamus. In this connexion it is interesting to note that after reserpine a typical picture of rage is not obtained (Schneider, 1955). In sham rage experiments, the influence of anaesthetics is absent. It appears that the effects of reserpine are due not only to selective depression of sympathetic centres in the diencephalon but also to facilitation of parasympathetic areas. The active facilitation of parasympathetic areas may also explain the production of bradycardia, miosis, etc., as well as the aggravation of existing bronchial asthma, renal colic, biliary colic, and ulcerative colitis which are observed in some hypertensive patients after reserpine treatment, as reported by Vakil (1954) and by Doyle and Smirk (1955).

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References

Barrett, W. E., Rutledge, R. A., and Rogie, B. (1954). *Fed. Proc.*, **13**, 334.
Bein, H. J. (1953). *Experientia*, **9**, 107.
Bhargava, K. P., and Borison, H. L. (1955). *J. Pharmacol.*, **115**, 464.
Delgado, J. M. R., and Anand, B. K. (1953). *Amer. J. Physiol.*, **172**, 162.
Doyle, A. E., and Smirk, F. H. (1955). *Practitioner*, **174**, 135.
Harrison, F., and Goth, A. (1955). *Fed. Proc.*, **14**, 349.
Ray, G. K., Roy, P. K., Dasgupta, S. R., and Werner, G. (1953). *Arch. exp. Path. Pharmak.*, **219**, 310.
Schneider, J. A. (1955). *Amer. J. Physiol.*, **181**, 64.
Trapold, J. H., Plummer, A. J., and Yonkman, F. F. (1954). *J. Pharmacol.*, **110**, 205.
Tripod, J., and Meier, R. (1954). *Arch. int. Pharmacodyn.*, **97**, 251,
Vakil, R. J. (1954). *Lancet*, **2**, 726.

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PHARMACOLOGICAL EFFECTS PRODUCED BY INTRACEREBRAL INJECTION OF DRUGS IN THE CONSCIOUS MOUSE

BY

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A method has been described for the study of the central effects produced by the intracerebral injection of drugs in the unanaesthetized mouse. The effects observed were in good agreement with those obtained after similar injections in cats, dogs and human beings. After intracerebral injection, drugs of diverse structure produced certain generalized effects: changes in positioning of the tail, stupor, hyperexcitability and tachypnoea. Both acetylcholine and methacholine produced an akinetic seizure and depression, but the latter compound also caused lacrimation and salivation. Atropine produced piloerection, increased sensitivity to sound and touch, clonic convulsions and scratching, whereas hexamethonium caused Parkinsonian-like muscle tremors and peripheral vasodilatation. After adrenaline, hyperexcitability, exophthalmos, stupor and death from pulmonary oedema were observed, but (+)-methylamphetamine produced only piloerection and exaggerated activity in response to sound and touch. Ergotamine caused a decreased sensitivity to sound and touch, micturition, and stupor, while ergometrine caused clonic convulsions, piloerection, defaecation and stupor.

There is much current interest in the pharmacological effects observed after the administration of drugs into the brain of conscious animals. Such procedures give a better estimation of the central actions of drugs because diffusion through the blood-brain barrier is not involved. Furthermore, the doses required to produce an effect by this mode of administration are, in general, much less than those required by other routes. Feldberg and Sherwood (1953a and b, 1954a and b, 1955) have approached the problem by injecting various drugs into the lateral ventricles of cats by an implanted cannula. A similar technique has been used in dogs (Haley and Weinberg, 1955; Haley and Dickinson, 1956; Haley and McCormick, 1956). However, such animal preparations are too expensive for routine screening experiments, although they are useful for exploring sites of central activity as distinguished from peripheral ones. As a possible solution for the problem of screening for central activity, we have devised a simple method using direct intracerebral injection in the mouse.

METHODS AND MATERIALS

Two hundred and fifty mice (strain CF 1) of either sex, weighing 20 to 25 g. each, were used. Physiological saline solutions of the drugs studied were injected into groups of ten mice at each dose used. The animal was grasped firmly by the loose skin behind the head. The skin was pulled taut. A $\frac{1}{2}$ in., 27 gauge hypodermic needle attached to a 0.25 ml. syringe was inserted perpendicularly through the skull into the brain and 0.01 to 0.05 ml. of solution was injected. The site of injection was 2 mm. from either side of the midline on a line drawn through the anterior base of the ears (Fig. 1). For ascertaining the areas in the brain ventricular system into which the drugs penetrated, 0.05 ml. of a 1:10 dilution of Indian ink was injected, and the brains were sectioned and studied histologically. All drugs and control injections were tested in groups of ten animals.

RESULTS

The site of injection (Fig. 1) was critical because penetration into the ventricles was not accomplished by injecting further rostrally and too much damage to vital centres was produced by injecting more caudally. The size and length of the needle was also critical for the same reasons and, in addition, an increase in length resulted in extensive brain damage from movement during injection.

The distribution of the carbon particles in Indian ink injected into the ventricular system with this technique showed that drugs, given by

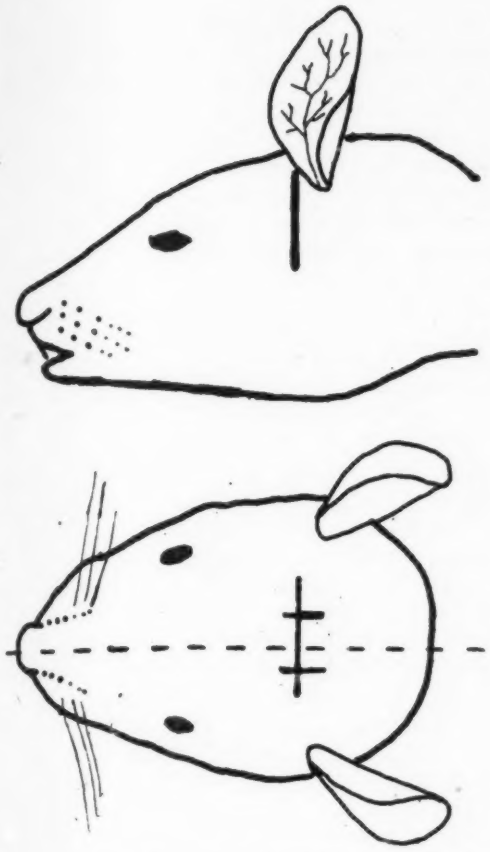

FIG. 1.—External landmarks for locating site of injection.

this route, could be expected to influence the activity of the vital centres located in the walls of the ventricles. Neuronal links between these and other centres in the brain might modify the responses observed. Passage of some of the injected material through the foramina of Luschka and Magendie might produce still other effects.

Insertion of the needle or injection of 0.01 to 0.05 ml. of physiological saline solution had a slight effect on the mice. Immediately after removal of the needle, the animals remained quiet for approximately one minute and then resumed their normal activity. None of the control animals showed any residual or detrimental effects from the procedure.

Acetylcholine.—Immediately after the intracerebral injection of 1 μg. of acetylcholine, the mice assumed a hunched-up position with their hind legs spread far apart. The animals appeared dazed and remained in this fixed position for two minutes. The position resembled an akinetic seizure. The Straube tail phenomenon was also observed at this time. The animals were highly excitable and would move rapidly if touched. If left alone, they remained in one position and appeared to be depressed. This latter condition lasted 10 to 15 min., after which the animals resumed their normal pre-injection activity. When the dose was increased to 10 μg. the above effects were more pronounced and the depressed condition lasted 30 to 40 min. The mice showed no residual effects 24 hr. later.

Methacholine.—Immediately after administration of 1 μg. of methacholine, the respiration was slowed and lacrimation and salivation were observed. The tail relaxed and remained parallel to the body. A depression of 10 to 15 min. duration was seen. An akinetic seizure similar to that seen with acetylcholine also occurred. When the dose was increased to 10 μg., the same effects were produced, but the depression appeared to be greater though its duration was still only 15 to 20 min. After both doses, the mice resumed their normal activity when the depression passed.

Atropine.—Intracerebral injection of 10 μg. of atropine caused increased respiration, a relaxation of the tail and a flattening of the ears against the head. Although the mice appeared depressed, they were extremely sensitive to both sound and touch. A weak stream of air would cause the animals to jump vertically several inches. Immediately after the injection of 100 μg. of atropine the mice had tachypnoea and piloerection. This was followed by bradypnoea and a generalized depression of 3 to 5 min. duration. During the next 15 to 30 min. the animals had clonic seizures and were extremely sensitive to sound and touch. A generalized itching sensation was also present because the animals scratched their bodies and particularly their noses. Fatigue and depression, lasting 2 to 3 hr., followed. Increasing the dose of atropine to 200 μg. decreased the primary depression to 2 min. and increased the period of clonic seizures to 30 to 45 min. and the secondary depression to 3 to 4 hr. All animals appeared normal 24 hr. later.

Hexamethonium Chloride.—A 1 μg. dose of hexamethonium produced tachypnoea, a relaxation of the tail and a drawing back of the ears. After a 15 to 30 min. period of depression, the mice resumed their pre-injection activity. Similar effects were observed after a 10 μg. dose, but the depression lasted 3 to 4 hr. During this period, the mice responded to sound or touch with generalized muscle tremors resembling those seen in a Parkinsonian syndrome. In addition, there was

a marked dilatation of the blood vessels of the legs and tail, and the latter was hot to the touch in contrast to the cold tails of the uninjected control animals. Increasing the dose to 50 μg. resulted in the same response seen after the 10 μg. dose. None of the animals died or showed any evidence of residual effects 24 hr. later.

Adrenaline.—Injection of 1 μg. of adrenaline caused an immediate increase in respiration, hyperexcitability and exophthalmos. The tail was elevated perpendicular to the body. After 15 min. the mice became sedated and could not be aroused. Normal activity partially returned in 30 to 40 min., but complete recovery required 5 to 8 hr. A 10 μg. dose produced an increase in respiration and a Straube tail phenomenon, followed shortly by stupor and death from pulmonary oedema. During the development of the oedema, the respiratory movements became weaker and gradually ceased.

(+)-*Methylamphetamine.*—Intracerebral injection of 100 to 200 μg. produced a rapid respiration, piloerection, a Straube tail phenomenon and exaggerated activity in response to sound and touch. All of these effects lasted from 1 to 4 hr. No residual effects were present 24 hr. later.

Ergotamine.—A 1 μg. dose of ergotamine caused tachypnoea, a flattening of the ears against the skull and a Straube tail phenomenon. Later the tail became straightened out behind the animal. A slight stupor with decreased response to touch and sound was also seen. Normal activity resumed in 10 to 15 min. Injection of 5 μg. produced tachypnoea followed by bradypnoea, urination, ears flattened against the head and a relaxed straight tail. The stuporous state was more pronounced, but the animal resisted attempts to change its position. There was a slight response to touch but not to sound. Normal activity returned in 30 to 40 min. and there was no detectable residual changes in the animals 24 hr. later.

Ergometrine.—An injection of 1 μg. of ergometrine produced the following effects: tachypnoea followed by bradypnoea, ears flattened against the head, a relaxed tail and stupor. The animals responded to sound with a clonic seizure. 20 to 45 min. later, normal activity was resumed. The same effects were produced by a dose of 5 μg., but, in addition, piloerection, defaecation and a Straube tail phenomenon were observed. There was only a slight response to stimulation, but the animals showed normal responsiveness 15 to 20 min. later. They appeared normal the next day.

Discussion

Depression similar to that described here in mice has been reported previously in cats (Dikshit, 1935; Silver and Morton, 1936; Bornstein, 1946; McCulloch, Ridley, and Sherwood, 1952; Feldberg and Sherwood, 1954a) and in man (Henderson and Wilson, 1936) receiving intracerebral injections of acetylcholine. The akinetic state was observed in cats by Feldberg and Sherwood (1954a).

The effects of atropine in the mouse were similar to those reported in the cat by Feldberg and Sherwood (1954a). Although no fatalities occurred, the higher doses presented a typical picture of the central effects usually observed after toxic doses of atropine.

The effects observed after intracerebral administration of hexamethonium in mice were in many ways similar to the responses obtained by Feldberg and Sherwood (1954a) in the cat. However, the peripheral vasodilatation was not observed in cats, and this is difficult to explain in terms of a block of transmission in peripheral ganglia. On the other hand, vasodilatation could be caused by a direct effect on the vasomotor centre.

A condition resembling sleep was the characteristic effect produced by the intracerebral administration of adrenaline in mice. A similar effect has been seen in dogs (Bass, 1914; Leimdorfer and Metzner, 1949), in cats (Feldberg and Sherwood, 1954a) and in human beings (Leimdorfer, Arana, and Hack, 1947; Leimdorfer, 1948). The fatal pulmonary oedema following central administration of adrenaline appeared to be characteristic of the rodent and has been reported earlier by Cassen and Kistler (1954).

Hyperexcitability also appeared to be a characteristic response to sympathomimetic amines because both adrenaline and methylamphetamine produced this effect. The longer duration of action of the latter compound following intracerebral injection was in accord with the results obtained by other routes of administration (Haley, 1947).

The decreased motor activity of the mice after ergotamine was similar to that reported in cats after intraventricular injection of ergotamine (Schwartz, Wakin, Bickford, and Lichtenheld, 1956). A stuporous state seemed to be the characteristic response produced in mice after intracerebral injection of ergot alkaloids. A similar response has been seen after the administration of these drugs into the third ventricle in cats (Goodman and Gilman, 1955). The clonic convulsions produced by ergometrine appeared to

be a characteristic action of this alkaloid because ergotamine did not produce this effect in the same dose.

Screening techniques require the establishment of the validity of the procedure in relation to what is already known concerning the particular drug(s) and function(s) being studied. Difficulties are attached to the study of centrally induced peripheral effects where the species under consideration is of the rodent family, since certain functions or reflexes are absent, such as the emetic reflex. However, valid results can be obtained if such differences in neurological development are borne in mind. There can be little doubt, from the data presented, that the intracerebral injection technique in the mouse can be used to study centrally induced peripheral effects. The mouse responses to drugs were, in the main, similar to those in other species, including human beings. The rapidity of response after intracerebral injection precluded any direct action on the peripheral receptors. However, there was the possibility, as pointed out earlier by Bedford (1953), that leakage of the drug through the needle track followed by absorption from the subarachnoid space into the systemic circulation could take place. There was also the fact that the volume injected, 0.01 to 0.05 ml., was greater than the volume of the cerebrospinal fluid. Thus, if the drug leaked out of the foramina and passed over the external surface of the brain, it might activate many other sites of nervous activity by a direct action. Under these circumstances absorption into the systemic circulation could take place. However, the amount of drug involved would seem to mitigate against such a possibility, particularly when one considers the dosage differential (20 to 40 times) required to produce pharmacological effects after parenteral administration.

Aside from its value as a screening procedure, the method could be used for student demonstration of the direct effects of drugs on the central nervous system in the absence of direct peripheral effects. It could also illustrate the possibility of centrally induced side effects. However, as many drugs do not pass the blood-brain barrier, the demonstration of centrally induced effects under such circumstances may be only of academic interest.

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References

Bass, A. (1914). *Z. ges. Neurol. Psychiat.* **26**, 600.
Bedford, T. H. B. (1953). *J. Physiol.*, **120**, 62P.
Bornstein, M. B. (1946). *J. Neurophysiol.*, **9**, 349.
Cassen, B., and Kistler, K. (1954). *Amer. J. Physiol.*, **178**, 53.
Dikshit, B. B. (1935). *J. Physiol.*, **83**, 42P.
Feldberg, W., and Sherwood, S. L. (1953a). Ibid., **120**, 3P.
—— —— (1953b). Ibid., **120**, 12P.
—— —— (1954a). Ibid., **123**, 148.
—— —— (1954b). Ibid., **125**, 488.
—— —— (1955). *Brit. J. Pharmacol.*, **10**, 371.
Goodman, L. S., and Gilman, A. (1955). *The Pharmacological Basis of Therapeutics*, 2nd ed., p. 581. New York: Macmillan.
Haley, T. J. (1947). *J. Amer. Pharm. Assoc., Sci. Ed.*, **36**, 361.
—— and Dickinson, R. W. (1956). Ibid., **45**, 432.
—— and McCormick, W. G. (1956). *Fed. Proc.*, **15**, 433.
—— and Weinberg, S. J. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 345.
Henderson, W. R., and Wilson, W. C. (1936). *Quart. J. exp. Physiol.*, **26**, 83.
Leimdorfer, A. (1948). *Wien. klin. Wschr.*, **60**, 382.
—— Arana, R., and Hack, M. A. (1947). *Amer. J. Physiol.*, **150**, 588.
—— and Metzner, W. R. T. (1949). Ibid., **157**, 116.
McCulloch, W. S., Ridley, E., and Sherwood, S. L. (1952). *Nature, London*, **169**, 157.
Schwartz, B. E., Wakin, K. G., Bickford, R. G., and Lichtenheld, F. R. (1956). *Arch. Neurol. Psychiat. Chicago*, **75**, 83.
Silver, G. A., and Morton, H. G. (1936). *J. Pharmacol.*, **56**, 446.
Weinberg, S. J., and Haley, T. J. (1955). *Circulation Research*, **3**, 103.
—— —— (1956). *Arch. int. Pharmacodyn.*, **105**, 209.

Brit. J. Pharmacol. (1957), **12**, 16.

THE INHIBITORY ACTION OF SOME ANTIMALARIAL DRUGS AND RELATED COMPOUNDS ON THE HEXOKINASE OF YEAST AND OF *PLASMODIUM BERGHEI*

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Of various antimalarial compounds tested, only proguanil failed to inhibit yeast hexokinase. The metabolite of proguanil, 10,580, was an effective inhibitor. Some compounds tested which were without antimalarial activity were potent inhibitors of yeast hexokinase. The degree of inhibition increased as the time during which the enzyme had been in contact with the drug increased, and the inhibitory action of mepacrine was reduced when the concentration of ATP was raised. The inhibition of yeast hexokinase by 10,732 was independent of the concentration of ATP.

The hexokinase of haemolysates of the reticulocytes of mouse or rat blood was not appreciably higher than that of similar haemolysates of normal erythrocytes. Preparations of mouse or rat erythrocytes parasitized with *P. berghei* possessed a much higher hexokinase activity.

The inhibiting action of various compounds on the hexokinase of *P. berghei* closely resembled those with yeast hexokinase. Again all antimalarial compounds (apart from proguanil) inhibited the enzyme, but some of the most potent inhibitors were devoid of antimalarial action. Amongst the chemotherapeutically active compounds, there appeared to be an approximate parallelism between antimalarial activity and potency as inhibitors of plasmodial hexokinase. The action of mepacrine on plasmodial hexokinase was reduced by raising the concentration of ATP, but, as with yeast hexokinase, the inhibition by 10,732 was independent of the ATP concentration.

From a consideration of the results, it seems doubtful whether this type of inhibitory effect plays more than a minor part in the mechanism of antimalarial action *in vivo*.

It has been reported that certain antimalarial drugs, including mepacrine and quinine, have an inhibitory action on various enzymes of the avian malarial parasite *P. gallinaceum* (Speck and Evans, 1945 ; Marshall, 1948 ; Moulder, 1949). It has been suggested that the inhibitory action of these drugs on the enzymes of the parasite might be related to their chemotherapeutic activity, and further investigation appeared desirable. The present work has been mainly concerned with the enzyme hexokinase, on which the metabolism of glucose by the parasite primarily depends.

As experiments with human malarial parasites were not practicable, it appeared desirable to use some other mammalian parasite, rather than the avian plasmodium. *P. berghei*, which infects small rodents, including mice and rats, was an obvious choice. In order to determine the best conditions for performing the inhibition experiments, preliminary studies were carried out with a partially purified sample of yeast hexokinase. These results afforded an interesting comparison with the analogous results obtained later with the enzyme of the malarial parasite.

The main object of the work was to ascertain whether any connexion appeared to exist between the antimalarial activity of the drugs and their inhibitory action on the enzyme. It was therefore decided to include in the experiments a number of compounds devoid of antimalarial action so that their anti-hexokinase activity might be determined.

It was suggested by Bovarnick, Lindsay, and Hellerman (1946) that mepacrine might interfere with the phosphorylation of glucose in malarial parasites by competition with adenosinetriphosphate (ATP), and Work and Work (1948) pointed out that the possibility could not be excluded that mepacrine acted by combining with ATP. Experi-

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ments were therefore carried out to investigate this possibility, and to elucidate the mechanism whereby certain of the drugs inhibited the enzyme.

Materials and Methods

Adenosinetriphosphate

ATP was isolated as the barium salt by the method of Dounce, Rothstein, Beyer, Meier, and Freer (1948). The barium ATP was converted into the potassium salt, a 5% excess of the theoretical amount of K_2SO_4 being used, and was stored at a final concentration of 0.05 M at −15° C.

Yeast Hexokinase

Hexokinase was prepared according to the procedure of Slater (1953) after autolysis of fresh baker's yeast by the method of Allfrey and King (1950). It was stored as a paste at −15° C., and retained its high activity over a period of three years. This active preparation was highly diluted before use, so that in any one experiment the concentration of $(NH_4)_2SO_4$ was negligible; therefore the preparation was not dialysed before use.

Plasmodial Hexokinase

A haemolysate of blood from mice infected with *P. berghei* was used as a source of hexokinase from the malarial parasite. The original strain of *P. berghei* was obtained from Dr. D. G. Davey, Imperial Chemical (Pharmaceuticals) Ltd. Young animals were more easily infected than those which were more than three months old, and so mice weighing 18–22 g. were used. They were inoculated intraperitoneally with 30 to 40 million red cells infected with the parasite, and were anaesthetized with chloroform about 3½ days later at the peak of parasitaemia. About 0.5 ml. blood was obtained from each mouse by cardiac puncture. Blood haemolysates were prepared by a slight modification of the method used by Speck and Evans (1945), who restored the salt concentration of the haemolysate to 0.9%. This was found to be unnecessary, and even inhibitory, and was omitted.

Antimalarial Drugs and Related Compounds

The compounds listed below were examined.

Number	*Chemical name*
5741	N^1-*o*-chlorophenyl-N^5-*iso*propylbiguanide HCl
5943	N^1-3 : 4-dichlorophenyl-N^5-*iso*propylbiguanide HCl
10,580	4 : 6-diamino-l-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine HCl
10,732	4 : 6-diamino-1-(3 : 4-dichlorophenyl)-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine HCl
5109	4-amino-6-*p*-chloroanilino-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine
15,587/B	4-amino-6-(3 : 4-dichloroanilino)-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine
6740/B	3-methoxy-8-chloro-5-(7-diethylaminoheptylamino)-acridine 2HCl
5068/B	2 : 3-dimethyl-4-(3-diethylaminopropylamino)-quinoline 2HCl

Determination of Hexokinase Activity

Method A.—The reaction mixture was buffered at *p*H 7.6 with aminotrishydroxymethylmethane (tris) (Gomori, 1946) or potassium phosphate with a final concentration of 0.0105 M, and normally contained: glucose, 0.00125 M; ATP, 0.004 M; $MgCl_2$, 0.005 M; drug, 0.004 M or distilled water. Diluted solution of stock yeast hexokinase preparation (0.1 ml.) or blood haemolysate (0.2 ml.) was added; the total volume was 1.2 ml. The enzyme underwent preliminary incubation with the drug for 15 min. at 20° C., or with one of its substrates and the drug at 30° C. The remaining substrates were then added, and the mixture was incubated for a further 15 min. at the same temperature as before. The reaction mixtures were deproteinized with $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945), and glucose was determined in the filtrates by the copper reduction method of Nelson (1944).

Method B.—Hexokinase activity was measured in some experiments by the Warburg manometric technique. The system was buffered with $NaHCO_3$ in an atmosphere of N_2+CO_2 (95 : 5). At 37° C. under these conditions, a concentration of 0.02 M-$NaHCO_3$ is required to maintain a *p*H of 7.6. The glucose-6-phosphate produced in the hexokinase reaction released CO_2 from the bicarbonate, and the enzyme activity was measured by the production of CO_2 in µl. The reaction mixture normally contained: glucose, 0.005 M; ATP, 0.004 M; $MgCl_2$, 0.005 M; drug, 0.004 M; $NaHCO_3$, 0.02 M; and diluted stock yeast hexokinase preparation (0.2 ml.); the total volume was 1.2 ml. The flasks were equilibrated for 20 min. at 37° C., one of the substrates in water containing 0.02 M-$NaHCO_3$ being in the side-arm. This period also served as a preliminary incubation period for the enzyme-inhibitor complex. The reaction was started by tipping in the substrate from the side-arm and allowed to proceed for a further period (usually 30 min.), readings being taken at 5–10 min. intervals.

Determination of Inorganic Phosphate

Inorganic phosphate was determined by the method of Berenblum and Chain (1938) as modified by Long (1943).

Production of Reticulocytosis

Mice of 20 g. and rats of 100 g. were treated with acetyl phenylhydrazine for 8 to 10 days. They received a daily intraperitoneal injection of 0.2 ml. normal saline containing 2 mg./ml. acetyl phenylhydrazine for each 100 g. body weight. The blood of the animal was withdrawn by heart puncture 48 hr. after the last injection. Microscopic examination following staining with cresyl blue showed that, both in rats and mice, 70–80% of the red cells were present as reticulocytes.

Results

Stability of Partially Purified Yeast Hexokinase Preparations

The partially purified yeast hexokinase preparation which was used in this work underwent ther-

mal inactivation when incubated in the absence of its substrates at temperatures of 30° C. and over, and at a *p*H of 7.6. Since the experimental method involved the incubation of the enzyme with the drug before the start of the enzymic reaction, the degree of thermal inactivation of the enzyme during this preliminary period was investigated. The yeast hexokinase preparation in a dilution of 1:2,500 was incubated at 30° C. in phosphate buffer at *p*H 7.6 for 30 min., either alone or in the presence of ATP 0.004 M, glucose 0.00125 M, versene (ethylenediaminotetraacetic acid) 0.001 M, or cysteine 0.004 M. All four compounds increased the stability of the enzyme ; the amount of glucose used in 15 min. was about three times as much as that used by the enzyme after incubating alone.

The thermal inactivation of the enzyme when incubated alone at 30° C. was lowest when the enzyme preparation was highly diluted, a dilution of 1:1,500 retaining considerably more activity than one of 1:200 and 1:600. The dilution after addition of substrates was adjusted to 1:2,500. Further, it was shown that at a final dilution of 1:3,000 the curve of enzyme activity against time was almost linear from 5 to 55 min., which suggests uniform enzyme activity during this period. At a dilution of 1:2,500 the enzyme did not lose its activity for periods up to 1 hr. on incubation at 20° C. when buffered to *p*H 7.6 with tris 0.0105 M or K phosphate buffer 0.0105 M (Table I). Some experiments on yeast hexokinase

TABLE I

EFFECT OF TEMPERATURE ON THE RATE OF INACTIVATION OF YEAST HEXOKINASE AFTER PRELIMINARY INCUBATION WITH TRIS AND WITH PHOSPHATE BUFFERS

Yeast hexokinase 0·1 ml. was first incubated with tris 0·0105M or phosphate 0·0105M *p*H 7·6, for various times at 20, 30, and 37° C. ATP 0·004M, glucose 0·00125M, and $MgCl_2$ 0·005M were then added; total vol. 1·2 ml. Incubated for 15 min. at 30° C. Final dilution stock yeast hexokinase preparation was 1: 2,500. Initially 1·5 μmole glucose was present in each sample.

Buffer	Preliminary Incubation Time (min.)	Glucose Used (μmoles)		
		20° C.	30° C.	37° C.
Tris ..	0	1·25	1·25	1·31
	15	1·36	1·06	0
	30	1·36	0·72	0
	60	1·42	0·53	0
Phosphate	0	1·23	1·25	1·28
	15	1·28	1·04	0
	30	1·35	0·80	0
	60	1·40	0·59	0

and all experiments on plasmodial hexokinase were carried out at 20° C. in the presence of tris or K phosphate buffer at *p*H 7.6. A considerable number of experiments on yeast hexokinase was carried out at 30° C. or 37° C. before the advantage of working at 20° C. had been appreciated.

The Inhibition of Yeast Hexokinase by Antimalarial Drugs and Related Compounds

Mepacrine, chloroquine, 10,580, 10,732, and quinine, all active antimalarial drugs, inhibited the action of yeast hexokinase to some extent, but not all inhibiting compounds were antimalarial drugs (Table II). Thus 5109, an isomer of 10,580, and 15,587/B, an isomer of 10,732, although they had

TABLE II

INHIBITION OF YEAST HEXOKINASE BY ANTIMALARIAL DRUGS AND RELATED COMPOUNDS

The numerals in brackets indicate the range of results. In method B the results given are for preliminary incubation for 20 min. at 37° C. of (a) enzyme with drug and ATP: glucose in the side-arm, and of (b) drug with ATP: enzyme and glucose in side-arm.

Drug	Method A. Av. % Inhibition	No. of Expts.	Method B. Av. % Inhibition		
			(a)	No. of Expts.	(b)
Proguanil ..	0		0		—
6740/B ..	0		0		—
5741 ..	—		10 (—)	2	—
5068/B ..	—		16 (12–19)	6	—
Quinine ..	—		18 (13–20)	6	—
Mepacrine..	18 (16–22)	6	23 (16–30)	8	8
10,580 ..	—		20 (18–23)	8	5
10,732 ..	20 (17–23)	6	22 (19–24)	8	5
Chloroquine	31 (30–33)	8	32 (30–37)	6	10
5109 ..	—		41 (36–44)	6	—
15,587/B ..	73 (—)	2	70 (65–73)	8	13

no antimalarial activity, had a very powerful inhibiting action. Proguanil had an effect on the estimation of glucose by reducing the amount of colour produced by the Nelson reagents. The difficulty was overcome by the use of appropriate controls containing proguanil, and it was tentatively concluded that proguanil was unable to inhibit yeast hexokinase. This conclusion was confirmed by results obtained with the Warburg manometric technique.

The Effect of Various Factors on the Degree of Inhibition of Yeast Hexokinase by Antimalarial Drugs and Related Compounds

Time of Addition of Drug.—The percentage inhibition was much less when the drug was in contact with ATP during the preliminary incubation period than when it was in contact with the enzyme during this period (Table III). Also, the degree of inhibition increased progressively, and was determined by the total time that the drug had been in contact with the enzyme, regardless of whether the drug was added at the beginning of the preliminary incubation or at some later stage in the reaction. These results suggested that the drug was acting primarily on the enzyme and not on ATP.

Concentration of ATP.—The degree of inhibition of yeast hexokinase by mepacrine and 10,732

TABLE III

EFFECT OF VARYING CONCENTRATIONS OF ATP ON THE INHIBITION OF YEAST HEXOKINASE BY MEPACRINE AND 10,732

Reaction medium: glucose 0·00125M; K phosphate buffer 0·01M, *p*H 7·6; $MgCl_2$ 0·005M; yeast hexokinase 0·1 ml.; total vol. 1·2 ml. Preliminary incubation for 15 min. at 30° C. with either glucose or ATP; reaction then allowed to proceed for 15 min. at 30° C. Final dilution stock yeast hexokinase preparation was 1: 2,500.

Drug	Final Concn. (M)	Concn. ATP (M)		% Inhibition
		During Preliminary Incubation	Added at Beginning Reaction	
Mepacrine	0·004	—	0·001	100
	0·004	—	0·002	88
	0·004	—	0·004	68
	0·004	—	0·008	25
	0·002	0·002	—	50
	0·004	0·002	—	76
	0·002	0·004	—	0
	0·004	0·004	—	0
10,732 ..	0·004	—	0·001	67
	0·004	—	0·002	69
	0·004	—	0·004	64
	0·004	—	0·008	61
	0·002	0·002	—	64
	0·002	0·004	—	64
	0·004	0·002	—	70
	0·004	0·004	—	74

in the presence of varying amounts of ATP is shown in Table III. When yeast hexokinase had undergone preliminary incubation in the presence of glucose and mepacrine, the inhibition produced was diminished if a large amount of ATP was added at the end of the preliminary incubation. However, when ATP and mepacrine, but not glucose, were present during the preliminary incubation the inhibition was not so great, and 0.004 M-ATP was sufficient to protect the enzyme completely. With 10,732 the percentage inhibition was almost independent of the concentration of ATP.

Concentration of Glucose.—When the concentration of glucose was raised from 0.00125 M to 0.0025 M there was no reduction in the inhibition by mepacrine. After preliminary incubation of yeast hexokinase for 15 min. at 30° C. with ATP 0.002 M and mepacrine 0.002 M and 0.004 M, glucose 0.00125 M or 0.0025 M was added. The inhibitory activity of mepacrine was no weaker at the higher concentration of glucose. However, in this type of experiment the amount of glucose could not be usefully increased to any great extent, because only a very small proportion of the total would then be utilized by the enzyme. In these circumstances, experimental errors in the determination of the percentage inhibition tend to become very great.

Further Studies at 20° C.—Certain of these results were confirmed at 20° C. (Table IV). At this temperature the enzyme does not undergo appreciable thermal inactivation within 3 hr., and so the action of inhibitors could be investigated in the absence of both glucose and ATP. With mepacrine at 20° C. the degree of inhibition increased when the preliminary incubation period was extended to 2 hr., but it tended to reach a limit after this time. The inhibitory power of mepacrine was again antagonized by ATP. On the other hand, the inhibitory potency of 10,732 was not affected by ATP. With low concentrations of this drug the degree of inhibition increased during the period of contact with the enzyme up to 1 hr., but longer incubation resulted in no further inhibition. With higher concentrations of 10,732 inactivation was more rapid, and rose steadily over the whole period (3 hr.) in which observations were made. Similar results were obtained with 15,587/B, which proved to be a powerful inhibitor of yeast hexokinase. With this compound a decrease in enzyme inhibition resulted from an increase in the ATP concentration, suggesting some degree of competition between the drug and ATP. K fluoride 0.0035 M did not affect the uitilization of glucose by yeast hexokinase either in the presence or absence of drugs under the conditions in Table IV.

TABLE IV

INHIBITORY EFFECT OF MEPACRINE, 10,732, AND 15,587/B ON YEAST HEXOKINASE AFTER INCUBATION FOR VARIOUS TIMES AT 20° C.

Reaction mixture: glucose 0·00125M; $MgCl_2$ 0·005M; K phosphate buffer 0·01M, *p*H 7·6; yeast hexokinase 0·1 ml.; total vol. 1·2 ml. Enzyme was first incubated with drug and phosphate at 20° C. Substrates were then added and the reaction proceeded for 7½ min. at 20° C. Final dilution of stock yeast hexokinase preparation was 1: 2,500.

		ATP	% Inhibition Observed on Preliminary Incubation for:				
			0	½	1	2	3 hr.
Mepacrine	0·001M	0·004M	0	9	18	26	26
,, ..	0·001M	0·002M	0	10	21	35	35
,, ..	0·002M	0·004M	0	26	26	41	41
,, ..	0·002M	0·002M	0	30	30	50	50
10,732 ..	0·001M	0·004M	0	13	22	22	22
,, ..	0·001M	0·002M	0	13	22	22	22
,, ..	0·002M	0·004M	0	13	22	38	47
,, ..	0·002M	0·002M	0	16	25	34	44
15,587/B	0·001M	0·004M	0	14	38	59	59
,, ..	0·001M	0·002M	17	24	45	69	69
,, ..	0·002M	0·002M	28	59	90	90	90

P. berghei *Hexokinase and Erythrocyte Lysates*

Adenosinetriphosphatase and Glucose-6-phosphatase.—The presence of adenosinetriphosphatase and glucose-6-phosphatase in haemolysates of normal and parasitized blood might introduce an error into the estimation of hexokinase activity. When normal mouse blood haemolysate was incubated with ATP or with glucose-6-phosphate, there was no increase in the inorganic phosphate

content. It was concluded that the activity of these enzymes was negligible. When a haemolysate of parasitized mouse erythrocytes was incubated with glucose-6-phosphate, there was again no detectable increase in inorganic phosphate ; but on incubation with ATP 0.004 M at *p*H 7.6 and at 20° C., 1.43 μg. inorganic phosphate was liberated presumably by the action of ATP-ase. This increase in inorganic phosphate was prevented by the incorporation of KF 0.0035 M in the haemolysate.

The Hexokinase Activity of Haemolysates of Normal and Parasitized Erythrocytes.—The hexokinase activity of normal and parasitized erythrocyte haemolysates was determined at 20° C. and *p*H 7.6 (Table V). Both rat and mouse erythrocytes were used in these experiments and gave

TABLE V

HEXOKINASE ACTIVITY OF HAEMOLYSATES OF NORMAL AND PARASITIZED ERYTHROCYTES AND OF RETICULOCYTES

Reaction mixture: glucose 0·00125M; KF 0·0035M; $MgCl_2$ 0·005M; ATP 0·004M; tris 0·0105M, *p*H 7·6; haemolysate 0·2 ml.; total vol. 1·2 ml. Incubation for 15 min. at 20° C.

Type of Haemolysate	Animal	Glucose Used (μmoles)
Normal	Mouse	0·14–0·20
„	Rat	0·12–0·26
70–80% parasitaemia ..	Mouse	0·80–1·20
70–80% „	Rat	0·84–1·40
70–80% reticulocytosis ..	Mouse	0·16–0·24
70–80% „	Rat	0·10–0·30

similar results. The hexokinase activity of the parasitized haemolysates was very much greater than that of a haemolysate of normal cells.

Certain malarial parasites, including *P. berghei*, show a strong predilection for reticulocytes, and in malarial infections there is a high degree of reticulocytosis. Sherwood Jones, Maegraith, and Gibson (1953) have shown that the respiration of rat reticulocytes is many times greater than that of mature red cells. The increased hexokinase activity of parasitized blood haemolysates as compared with normal haemolysates might therefore be related to the high degree of reticulocytosis rather than to the degree of parasitaemia. Table V shows that the hexokinase activity of blood haemolysates of animals with a high degree of reticulocytosis (70–80%) was not significantly higher than that of normal blood haemolysates. The high hexokinase activity of haemolysates of malarial erythrocytes is thus almost certainly due to the parasites themselves.

The glucose content of a haemolysate of highly parasitized blood was extremely low, and seldom exceeded 5–10 μg. in 0.2 ml. haemolysate, which is equivalent to 5 mg. corpuscular glucose/100 ml. whole blood as compared with an average value of 36 mg./100 ml. for whole normal blood. In many cases the glucose content of the parasitized haemolysate was so low that it could not be detected.

Stability and pH Optimum of Plasmodial Hexokinase.—The plasmodial hexokinase underwent no significant loss in activity when kept at 20° C. for 1 hr., 0.95 μmoles glucose being used by the fresh enzyme and 0.91 μmoles after storage. The plasmodial enzyme was observed to have a broad optimum from approximately *p*H 6.9–8.1.

The Inhibition of Plasmodial Hexokinase by Antimalarial Drugs and by Certain Other Compounds.—The compounds selected for study included certain antimalarial drugs, a number of related compounds, and also several alkaloids, primary aromatic amines, quinoline, and the powerful detergent cetrimide. An attempt was made to test 5943 and pyrimethamine, but these were very sparingly soluble at *p*H 7.6. The results were broadly parallel with those obtained using yeast hexokinase (Table VI). Mepacrine and

TABLE VI

INHIBITION OF PLASMODIAL HEXOKINASE BY ANTIMALARIAL DRUGS AND CERTAIN OTHER COMPOUNDS

Reaction medium: glucose 0·00125M; ATP 0·004M; $MgCl_2$ 0·005M; KF 0·0035M; tris 0·0105M, *p*H 7·6; inhibitor 0·004M; haemolysate 0·2 ml. Total vol. 1·2 ml. The haemolysate was incubated with buffer and drug for 15 min. at 20° C. Substrates were then added, followed by 15 min. incubation at 20° C.

Compound	% Inhibition
Proguanil	0
6740/B	0
5741	5
5068/B	9
Azacrin	11
5109	20
Quinine	20
10,580	22
Chloroquine	26
10,732	29
Mepacrine	32·5
15,587/B	60
Aniline	0
o-Toluidine	0
Quinoline	11
Brucine	13
Morphine	15
Strychnine	30
Cetrimide	100

10,732 appeared to be relatively more powerful inhibitors of the plasmodial enzyme than of the yeast enzyme ; chloroquine was more active than either of these against yeast hexokinase. Strychnine was as potent an inhibitor of plasmodial hexokinase as mepacrine. Cetrimide proved to be the most powerful inhibitor of the enzyme. At a concentration of 0.002 M and under the conditions shown in Table VI, it inhibited plasmodial hexokinase to the extent of 50%.

The Effect of the Concentration of ATP on the Inhibition of Plasmodial Hexokinase by Mepacrine and 10,732.—From the results in Table VII it appeared that the inhibition of plasmodial hexokinase by mepacrine was the higher the lower the concentration of ATP, but with 10,732 the inhibition was not influenced by ATP in this manner. The results closely resembled those obtained with yeast hexokinase.

TABLE VII

EFFECT OF THE CONCENTRATION OF ATP ON THE INHIBITION OF PLASMODIAL HEXOKINASE BY MEPACRINE AND 10,732

Conditions as in Table VI

ATP (Final Concn. M)	% Inhibition Mepacrine	% Inhibition 10,732
0·004	30	30
0·002	40	29
0·001	57	35

DISCUSSION

The results presented indicate that the drugs inhibit yeast hexokinase by attacking the enzyme itself, rather than by combining with ATP. The degree of inhibition increases as the time during which the enzyme has been in contact with the drug is lengthened. The higher the concentration of ATP, the lower is the degree of inhibition. The inhibitory action of the drug, however, appears to be independent of the concentration of glucose.

These results can be partially explained if it is assumed that there are two types of active groups on the enzyme surface. Those of the first type are glucose acceptors. Glucose is not so effective at protecting yeast hexokinase as ATP, and, since the degree of inhibition appears to be independent of the glucose concentration, the mepacrine molecules presumably do not interfere at these sites. The active groups of the second type are ATP acceptors. When there is no ATP present during the preliminary incubation period the mepacrine molecules are able to occupy these sites to the exclusion of ATP, and inhibition results. If much ATP is added at the end of the preliminary incubation period, the degree of inhibition is reduced; this suggests that ATP can displace mepacrine from the sites, and so antagonize its action. If, on the other hand, the ATP is present during the preliminary incubation of the enzyme with mepacrine, fewer of the ATP acceptors are available for mepacrine and the resulting inhibition is less. The progressive nature of the inhibition may be due to a slow local modification of the structure of the hexokinase molecule when the ATP acceptors are occupied by mepacrine. This structural change may perhaps be regarded as a local denaturation of the protein. The ATP, on displacing the mepacrine, may be able to reverse this "denaturation" provided that it has not gone too far, and thus restore the original structure of the yeast hexokinase.

The results in Table V demonstrate that, in spite of the high respiration rate of rat and mouse reticulocytes compared with normal erythrocytes (Sherwood Jones *et al.*, 1953), there is no corresponding difference in hexokinase activity. Calculation shows that if all the observed oxygen uptake of the reticulocyte depends on the oxidation of glucose, and if this glucose is first phosphorylated under the influence of hexokinase, then the hexokinase present is amply sufficient to account for the respiration of the cells. Thus there is no inconsistency between the hexokinase results and those reported by Sherwood Jones *et al.* on the rate of oxygen uptake by reticulocytes. It would appear that the hexokinase activity of the reticulocytes remains almost intact in the mature erythrocyte, along with the rest of the activity of the glycolytic system, whereas the tricarboxylic acid cycle no longer functions. The oxygen uptake of the mature erythrocyte is therefore very small or non-existent.

It may be argued that it is preferable to separate the parasites from the haemolysate, so that the parasite enzymes may be studied without contamination with those of the erythrocytes. This, however, involves the use of a haemolytic agent such as saponin, and it appears advantageous, in the first instance at least, to adopt the simpler method of extracting the parasitized erythrocytes with distilled water, and thus avoid the use of surface active agents. The hexokinase activity of both normal erythrocytes and reticulocytes has been shown to be very small compared with that of the parasites. Any contribution in the haemolysates of parasitized cells made by the erythrocytes would presumably be significantly less than would be possessed by haemolysates of a comparable number of normal cells, in consequence of the physical presence of the parasites within the former.

It appears from Table II that all active antimalarial drugs inhibit yeast hexokinase, but certain compounds devoid of antimalarial activity have also an inhibitory effect. Tables II and VI show that there is close similarity between the results obtained with yeast hexokinase and those with the plasmodial enzyme.

The information on the chemotherapeutic action of the compounds against *P. berghei* is somewhat conflicting. From the review by Thurston (1953)

it appears that the most potent plasmodicidal effect is produced by pyrimethamine, which it was unfortunately impossible to investigate in the present work because of the very low solubility of it and its salts.

Though not so potent as mepacrine and chloroquine, proguanil is an effective agent against *P. berghei*, but has no inhibitory effect on hexokinase. It is generally agreed that proguanil itself has no significant antimalarial action, but is converted into the triazine, 10,580. This compound has a definite action on the enzyme, though less than that of mepacrine and chloroquine. This is in harmony with the weaker chemotherapeutic effect of proguanil compared with that of mepacrine or chloroquine, which are almost equally active against *P. berghei*. Unfortunately, there appears to be no information as to the relative efficacy of proguanil and its triazine metabolite against *P. berghei* in mice. Crowther and Levi (1953) found that 10,580 was 10 times as active as proguanil in suppressing *P. gallinaceum* infections in chicks. *P. berghei* is, however, less sensitive than *P. gallinaceum* to the action of proguanil. The results with the biguanyl compound, 5943, and its active triazine metabolite, 10,732, are in general agreement with these findings. Like proguanil, 5943 appeared to have little or no action on hexokinase, but precise results could not be obtained because of the very low solubility of the drug. It is, however, more potent as an antimalarial agent than proguanil, being approximately equal to mepacrine and chloroquine in its activity against *P. berghei*. In accordance with this, its metabolite, 10,732, has about the same anti-hexokinase activity as the latter two drugs.

Quinine has a very low antimalarial action against *P. berghei*, compared with mepacrine, but its inhibitory action on plasmodial hexokinase is about 20%, as compared with 32% for mepacrine under similar conditions. The chloroquine analogue, 5068/B, has only very slight antimalarial activity, which agrees well with its low inhibitory potency, and 6740/B, which completely failed to inhibit, is without antimalarial activity.

Thus it appears that all active antimalarial drugs tested, with the exception of proguanil, did inhibit plasmodial hexokinase, and there was at least an approximate correlation between their antimalarial activity and their inhibitory power. However, all inhibiting compounds were not chemotherapeutically active. Of the compounds related chemically to the series of antimalarial drugs, the most powerful inhibitor was 15,587/B, which is said to be completely inactive against avian malaria. 5109, which is also inactive, was as good an inhibitor as its active isomer, 10,580. Amongst other compounds tested on the hexokinase of *P. berghei*, several possessed appreciable inhibitory activity, including the alkaloids brucine, morphine, and strychnine. In view of the inhibition by these alkaloids, it is perhaps not surprising that quinine has an inhibitory action on plasmodial hexokinase higher than would be expected on the basis of its antimalarial activity. It is noteworthy that cetrimide had a higher anti-hexokinase activity than any other compound tested.

The inhibition of the enzyme by compounds devoid of antimalarial activity casts grave doubts on the validity of any theory of antimalarial action based on the inhibition of plasmodial hexokinase. It has always to be borne in mind, however, that many factors may deprive a compound of chemotherapeutic action *in vivo*, even although it may possess some of the essential properties for activity. There may be differences in the permeability of the erythrocyte membrane to the compound, or some mechanism, at present unknown, may render them inactive *in vivo*.

It is important to note that the concentrations of drugs required to produce an appreciable degree of inhibition are much higher than those required to bring about definite chemotherapeutic effects. This is a further argument against the view that the inhibition of the hexokinase system plays an important part in the action of antimalarial drugs. Nevertheless, in the intact animal, the drugs may be concentrated, either locally in various tissues, or in particular structures inside the cells, so that at those key sites the concentration of the drug may be very high.

It is possible that a really effective chemotherapeutic agent may exert several effects on parasite metabolism, some of which may be more essential than others, but which are complementary in destroying the organism. The antagonism of certain antimalarial agents to folinic acid may play a key role in establishing chemotherapeutic activity, but other functions, of which the anti-hexokinase activity may be one, may possibly fulfil a subsidiary role.

We want to thank Dr. D. G. Davey, Imperial Chemical (Pharmaceuticals), Ltd., for the original strain of *P. berghei*, and for much information on the maintenance of the malarial infection in white mice. Most of this work was carried out during the tenure by one of us (D. M. F.) of a grant from the Medical Research Council, to whom we are also most grateful. Our thanks are also due to Miss Iris Creighton for technical assistance.

References

Allfrey, V. G., and King, C. G. (1950). *J. biol. Chem.*, **182**, 367.

Berenblum, I., and Chain, E. (1938). *Biochem. J.*, **32**, 295.

Bovarnick, M. R., Lindsay, A., and Hellerman, L. (1946). *J. biol. Chem.*, **163**, 523, 535.

Crowther, A. F., and Levi, A. A. (1953). *Brit. J. Pharmacol.*, **8**, 93.

Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R., and Freer, R. M. (1948). *J. biol. Chem.*, **174**, 361.

Gomori, G. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 33.

Long, C. (1943). *Biochem. J.*, **37**, 215.

Marshall, P. B. (1948). *Brit. J. Pharmacol.*, **3**, 1.

Moulder, J. W. (1949). *J. infect. Dis.*, **85**, 195.

Nelson, N. (1944). *J. biol. Chem.*, **153**, 375.

Sherwood Jones, E., Maegraith, B. G., and Gibson, Q. H. (1953). *Ann. trop. Med. Parasit.*, **47**, 431.

Slater, E. C. (1953). *Biochem. J.*, **53**, 157.

Sols, A., and Crane, R. K. (1954). *J. biol. Chem.*, **206**, 927.

Somogyi, M. (1945). Ibid., **160**, 69.

Speck, J. F., and Evans, E. A. (1945). Ibid., **159**, 71, 83.

Thurston, J. P. (1953). *Exp. Parasitol.*, **2**, 311.

Work, T. S., and Work, E. (1948). *The Basis of Chemotherapy*. Edinburgh: Oliver and Boyd.

Brit. J. Pharmacol. (1957), **12**, 24.

THE EFFECT OF ESERINE AND NEOSTIGMINE ON THE BLOOD PRESSURE OF CONSCIOUS RATS

BY

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Small amounts of eserine salicylate (10 to 20 μg.) regularly caused a rise of blood pressure in non-anaesthetized rats. Neostigmine methylsulphate in doses from 1 to 10 μg./animal usually caused a fall of blood pressure, or no change was observed; only in a few experiments was a rise of blood pressure noted. The pressor effect of eserine was abolished by atropine and reduced or abolished by yohimbine and phentolamine. Adrenalectomy did not change the response to eserine. The present experiments do not contradict earlier statements that the pressor effect of eserine was due to the discharge of impulses from a centre or centres in the central nervous system.

Several workers have observed that eserine raises the blood pressure of rats anaesthetized with urethane (Dirnhuber and Cullumbine, 1955; Varagić, 1955; Hornykiewicz and Kobinger, 1956). This effect of eserine was attributed to the discharge of impulses from a centre or centres in the brain at points not lower than the medulla.

It is generally known that the effect of some substances on the blood pressure of the same animal may depend on the anaesthetic agent and on the manner of preparation. We therefore decided to test the effect of eserine in non-anaesthetized rats. The influence of hexamethonium, of adrenalectomy, of atropine, and of antisympathomimetic agents on this effect was also studied.

METHODS

Male and female albino rats of 125 to 200 g. were used. For the indirect measurement of the blood pressure in the rat, a tail plethysmograph, described in detail by Van Proosdij-Hartzema (1954), was used. This method permits repeated blood-pressure measurements over a long period. Its principle is to exclude the vessels of the tail from the circulation by means of an external pressure exerted on the proximal part of the tail by an occluding cuff. This pressure is gradually released, and the value at which the inflow of blood starts again is read on a mercury manometer. In most experiments, the temperature of the fluid in the plethysmograph was kept between 41 and 44° C.

The drugs tested were injected into the tail vein in a volume of 0.3 ml.; only cocaine hydrochloride was injected intraperitoneally.

RESULTS

Action of Various Anticholinesterase Agents.– The effect of eserine salicylate on the blood pressure of non-anaesthetized rats is shown in Table I. In this series of experiments 10 μg. eserine salicylate was injected into each animal (60 to 77 μg./kg.). This dose caused a rise of blood pressure ranging from 24 to 95 mm. and lasting 20 to 57 min. The rise of blood pressure reached its maximum 4 to 16 min. after the injection of eserine. The increase in blood pressure was 12 to 158% of the initial level.

In another group of three rats, 20 μg. eserine salicylate (101 to 153 μg./kg.) caused a rise in blood pressure ranging from 56 to 76 mm. The duration of the effect was 76 to 99 min.

TABLE I

THE EFFECT OF INTRAVENOUS ESERINE SALICYLATE (10 μG.) ON THE BLOOD PRESSURE OF NON-ANAESTHETIZED RATS

No. of Expt.	Initial Blood Pressure (mm. Hg)	Maximum b.p. after Eserine (mm. Hg)	Maximum Reached after (min.)	Duration of Effect (min.)	% Increase over Initial Level
1	58	96	11	57	65
2	66	112	12	41	69
3	60	155	7	45	158
4	64	128	15	30	100
5	85	131	16	—	54
6	49	100	12	24	104
7	94	118	4	29	12
8	60	106	16	55	76
9	66	123	7	54	86
10	123	147	8	29	19
11	72	110	5	57	53
12	75	118	11	20	57
Mean	82	120	10	40	71

The effect of neostigmine methylsulphate was tested in a group of 14 rats. In nine of them neostigmine in doses of 2 to 5 μg. (13 to 33 μg./kg.) caused a fall of blood pressure ; in three the same dose caused no change of blood pressure ; and in two rises of 17 and 25 mm. were observed.

Action of Hexamethonium.—In rats anaesthetized with urethane, large amounts of hexamethonium (C_6) (128 to 167 mg./kg.), given in one or two injections, abolished the pressor action of eserine in doses of 30 to 90 μg./kg. (Varagić, 1955). Dirnhuber and Cullumbine (1955) used even larger doses of C_6 (200 to 400 mg./kg.) to abolish the pressor effect of another anticholinesterase agent, sarin (isopropyl methylphosphonofluoridate).

In the present experiments, C_6 itself in a dose of 30 mg./kg. caused a fall in blood pressure ranging from 10 to 69 mm. and lasting 4 to 15 min., except for one out of nine experiments when the fall lasted more than 30 min. Similar effects were observed after 40 mg./kg. C_6. A dose of 50 mg./kg. C_6 caused either no change in blood pressure or a rise ranging from 20 to 30 mm. This dose of C_6 killed four out of nine animals. Van Proosdij-Hartzema and De Jongh (1955) found that 5 and 20 mg./kg. C_6, given orally, hardly influenced the blood pressure, whereas 40 mg./kg. caused a slight rise.

The pressor effect of eserine in non-anaesthetized rats was not greatly affected by C_6 in doses of 30 to 50 mg./kg. Both potentiation and reduction of the effect of eserine were observed after these doses of C_6. In Fig. 1 the dose of 10 μg. eserine caused a rise of blood pressure of 25 mm. Before

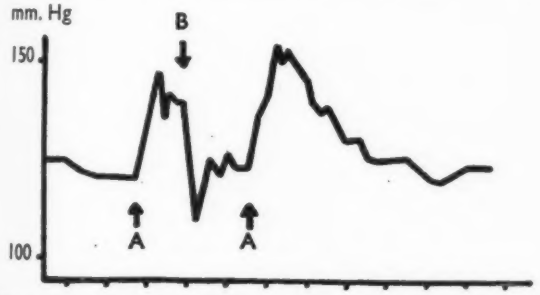

FIG. 1.—Rat, 165 g., no anaesthetic. The action of C_6 on the pressor effect of eserine. At A, 10 μg. eserine i.v. At B, 30 mg./kg. C_6 i.v. Note the potentiation of the pressor effect of eserine by C_6. Time in 10 min. intervals.

the effect of eserine had ceased, 30 mg./kg. C_6 was injected and it caused an abrupt fall in blood pressure. The injection of eserine was then repeated and it caused a rise of blood pressure of 30 mm. The opposite effect was obtained in another experiment. In this rat 10 μg. eserine caused a rise of blood pressure of 59 mm. When the pressure had returned to the initial level, 50 mg./kg. C_6 was injected and no change in blood pressure was observed. The repeated injection of 10 μg. eserine caused a rise in blood pressure of only 24 mm.

On the other hand, C_6 reduced the pressor effect of eserine if injected immediately after this drug. A typical experiment is shown in Fig. 2. In this rat 20 μg. eserine (117 μg./kg.) caused a rise of blood pressure of 61 mm. When the effect of eserine had reached a maximum, 30 mg./kg. of C_6 was injected. It caused a fall of blood pressure, but it did not completely abolish the effect of eserine, which was still evident 55 min. after the injection of C_6.

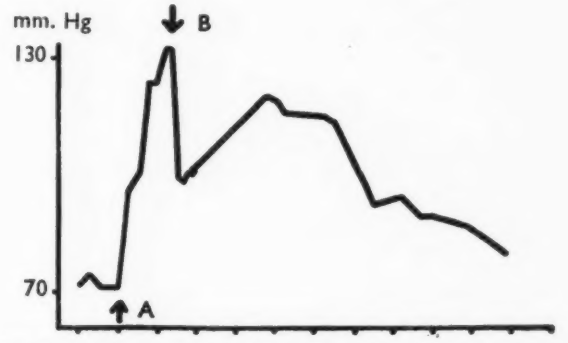

FIG. 2.—Rat, 170 g., no anaesthetic. The action of C_6 on the pressor effect of eserine. At A, 20 μg. eserine i.v. At B, 30 mg./kg. C_6 i.v. Time in 10 min. intervals.

Action of Eserine in Adrenalectomized Rats.—It has been known for a long time that eserine can produce a 10- to 15-fold increase in the output of adrenaline from the adrenals of the cat (Stewart and Rogoff, 1921). We thought that the pressor effect of eserine in the rat might be due to the liberation of adrenaline from the adrenals. A series of rats was accordingly adrenalectomized and, 9 to 18 days later, the effect of eserine on their blood pressures was tested. As will be seen from Table II, there was no significant difference between the duration of the effect in normal and in adrenalectomized rats.

The effect of C_6 in adrenalectomized rats was very similar to that observed in normal rats. Thus, in a group of three rats in which eserine was tested 8, 9 and 18 days after adrenalectomy, C_6 in a dose of 30 mg./kg. did not change the pressor effect of 10 μg. eserine. In another rat, 12 days after adrenalectomy, even 60 mg./kg. C_6, given in two injections, did not change the response to 10 μg. eserine. Only in one out of five experiments did

TABLE II

THE EFFECT OF INTRAVENOUS ESERINE SALICYLATE (10 μG.) ON THE BLOOD PRESSURE OF NON-ANAESTHETIZED ADRENALECTOMIZED RATS

No. of Expt.	Initial Blood Pressure (mm. Hg)	Maximum b.p. after Eserine (mm. Hg)	Maximum Reached after (min.)	Duration of Effect (min.)	% Increase over Initial Level
1	64	98	12	30	53
2	86	132	3	20	53
3	88	123	4	14	39
4	72	118	4	27	63
5	87	128	4	37	47
6	98	122	11	31	24
7	40	95	5	49	136
Mean	76	115	6	30	59

C_6 (30 mg./kg.) abolish the pressor effect of eserine 20 μg. (140 μg./kg.).

Abolition of Response by Atropine and Phentolamine.—Atropine sulphate in doses 2 to 7 mg./kg. completely abolished the pressor effect of eserine.

Yohimbine in doses of 5 to 10 mg./kg. distinctly reduced the effect of eserine, whereas phentolamine (Regitin) in doses from 7.5 to 30 mg./kg. reduced or even abolished the response to eserine, as shown in Fig. 3. Both phentolamine and yohimbine alone caused a fall of blood pressure.

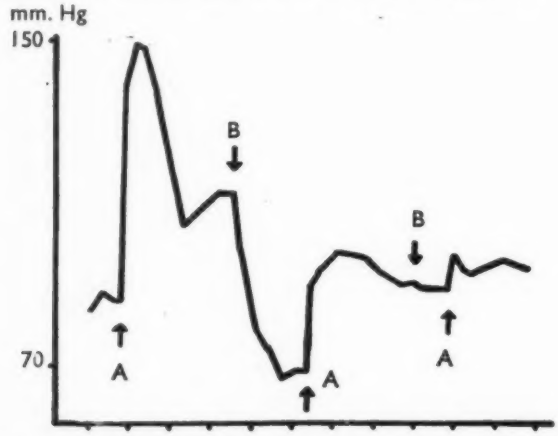

FIG. 3.—Rat, 150 g., no anaesthetic. The action of phentolamine on the pressor effect of eserine. At A, 20 μg. eserine i.v. At B, 10 mg./kg. phentolamine i.v. Note abolition of the effect of eserine by phentolamine. Time in 10 min. intervals.

Action of Cocaine.—It has been shown on rats under urethane that the pressor effect of eserine was increased after cocaine (Varagić, 1955). In non-anaesthetized rats measurement of the blood pressure by the method used in these investigations was impossible after intraperitoneal injection of cocaine (5 to 7 mg./kg.)—most probably because of constriction of the blood vessels in the tail.

DISCUSSION

The results obtained in the present experiments are in accord with the findings in the rat under urethane. In doses of 10 to 20 μg. (60 to 153 μg./kg.), eserine regularly caused a rise of blood pressure in non-anaesthetized rats. The previous studies on rats under urethane showed that the pressor effect of eserine was central in origin (Dirnhuber and Cullumbine, 1955 ; Varagić, 1955 ; Hornykiewicz and Kobinger, 1956), and that it was exerted at points not lower than the medulla. The present experiments do not contradict this view.

The pressor effect of eserine is not common to all anticholinesterase agents because neostigmine methylsulphate did not show this effect. Similar differences between eserine and neostigmine were observed by Eccles, Fatt, and Koketsu (1954) and Eccles, Eccles, and Fatt (1956) during pharmacological investigations on a central synapse at which acetylcholine was a transmitter. Thus, eserine greatly prolonged the duration of the discharge of impulses which an antidromic volley evoked from a Renshaw cell. In contrast to eserine, neostigmine was much less effective, even in a dose as large as 2 mg./kg. One possible explanation for the relative ineffectiveness that is usually observed for neostigmine given by intravenous injection is that a barrier to the diffusion of this quaternary amine surrounds the blood vessels of the central nervous system, whereas eserine, being a tertiary compound, can reach the central nervous system more readily. However, neostigmine would appear to be at least as effective as eserine in prolonging the discharge of impulses when applied to the environment of the Renshaw cell (Eccles *et al.*, 1956), or in causing a rise in blood pressure when injected intracisternally (Hornykiewicz and Kobinger, 1956).

The effect of eserine was not abolished by C_6 even in doses from 30 to 50 mg./kg., the last dose being close to the LD50 for our animals. In a rat under urethane, 128 to 160 mg./kg. C_6 had to be given in one or two injections in order to abolish the pressor action of eserine. It is difficult to explain why non-anaesthetized rats were more sensitive to C_6 than were rats under urethane. In non-anaesthetized rats the doses necessary to abolish the pressor effect of eserine could not be reached without killing the animals.

Liberation of adrenaline and noradrenaline from the adrenals does not seem to be responsible for the blood-pressure rise after eserine. The effect of eserine in the adrenalectomized rat was qualitatively the same as in the controls. It is

difficult to say whether the effect of eserine was easier to abolish by C_6 in the adrenalectomized than in the control rats. The fact remains that the only rat in which the effect of eserine was abolished after 30 mg./kg. C_6 was the adrenalectomized one. This might indicate that larger amounts of C_6 are necessary to block the adrenals than to block the sympathetic ganglia. On the other hand, the difficulty of blocking the effect of eserine by C_6 in normal rats might be due to potentiation by C_6 of the effect of small amounts of adrenaline and noradrenaline liberated from the adrenals.

Atropine sulphate in doses from 2 to 7 mg./kg. abolished the effect of eserine. The adrenaline antagonists yohimbine and phentolamine distinctly reduced or even abolished the effect of eserine. The effect of atropine does not seem to result from its ganglionic blocking action, because Cahen and Tvede (1953) found that in cats 6 to 15 mg./kg. atropine was necessary to block the ganglia controlling the blood pressure. Most probably, atropine acts centrally. Reduction or abolition of the effect of eserine by adrenaline antagonists would mean that the pressor effect of eserine was mediated by sympathetic impulses coming from the higher centres to the walls of the blood vessels.

References

Cahen, R. L., and Tvede, K. M. (1953). *Arch. int. Pharmacodyn.*, **94**, 248.

Dirnhuber, P., and Cullumbine, H. (1955). *Brit. J. Pharmacol.*, **10**, 12.

Eccles, J. C., Fatt, P., and Koketsu, K. (1954). *J. Physiol.*, **126**, 524.

—— Eccles, R. M., and Fatt, P. (1956). Ibid., **131**, 154.

Hornykiewicz, O., and Kobinger, W. (1956). *Arch. exp. Path. Pharmak.*, **228**, 493.

Van Proosdij-Hartzema, E. G. (1954). *Acta Physiol. Pharmacol. Neer.*, **3**, 472.

—— and De Jongh, D. K. (1955). Ibid., **4**, 160.

Varagić, V. (1955). *Brit. J. Pharmacol.*, **10**, 349.

Stewart, G. N., and Rogoff, J. M. (1921). *J. Pharmacol.*, **17**, 221.

Brit. J. Pharmacol. (1957), **12**, 28.

THE INHIBITION BY SODIUM SALICYLATE OF OEDEMA OF THE HIND-PAW OF THE RAT INDUCED BY 5-HYDROXYTRYPTAMINE

BY

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The subcutaneous administration of 600 mg./kg. body weight of sodium salicylate to adult rats 3 to 4 hr. previously inhibited the development of hind-paw oedema following the injection of 2.5 μg. of 5-hydroxytryptamine. This effect was more pronounced in normal than in adrenalectomized rats.

The inhibitory effect of large doses of salicylate on rat hind-paw oedema induced with testicular extract has been previously described (Kelemen, Majoros, Ivanyi, and Kovacs, 1950). Other types of oedema of the hind-paw, such as those produced by the histamine liberator 48/80, dextran, human sera with enhanced activity to induce oedema, or the animal protein permeability factor of Miles and Wilhelm (1955) were also inhibited by subcutaneous administration of salicylate. The present experiments are concerned with the inhibition of oedema induced with 5-hydroxytryptamine.

METHOD

Injection of 0.3 to 0.8 mg. of crude testicular extract, dissolved in 0.11 ml. saline, into the plantar side of the hind-paw of the rat caused a gross acute oedema. Each animal served as its own control immediately before injecting sodium salicylate (600 mg./kg.). The acute oedema reached its maximum within 10 to 20 min. Details of reading the grade of oedema formation have been given previously (Kelemen, 1956). The inhibition of oedema was recorded as "good" if no marked oedema developed around the tibia.

In the present experiments, 2.5 μg. of 5-hydroxytryptamine (Upjohn) was used to provoke topical oedema, by the same technique and with similar results, except for the slower effect of the 5-hydroxytryptamine. Similar observations on 5-hydroxytryptamine oedema have been made by Benditt and Rowley (1956). A dose of 0.5 to 1.0 μg. was also effective in our young rats.

RESULTS

Results of our attempts to influence this type of oedema with systemic sodium salicylate are presented in Table I. Sixty-eight rats of 165 to 200 g. body weight were used in this series. As shown, the 5-hydroxytryptamine oedema investigated 3 to 4 hr. after subcutaneous administration of 600 mg./kg. body weight of sodium salicylate was inhibited in the normal rat. A less pronounced inhibition was obtained with sodium salicylate in

TABLE I

INHIBITION OF TESTICULAR-EXTRACT-INDUCED AND OF 5-HYDROXYTRYPTAMINE OEDEMA OF THE HIND PAW BY SUBCUTANEOUS ADMINISTRATION OF 600 MG./KG. BODY WEIGHT OF SODIUM SALICYLATE IN THE NORMAL AND IN THE ADRENALECTOMIZED RAT

As untreated adrenalectomized rats usually died shortly after the administration of 600 mg./kg. of sodium salicylate, the groups of adrenalectomized animals marked thus * received two doses of 1 mg. of cortisone 8 to 18 days before being given sodium salicylate. This dose of cortisone was without effect on permeability.

	No. of Animals in Each Group	No. of Animals Showing Inhibition of Oedema Formation 3 to 4 Hr. after Sodium Salicylate		
		Good	Moderate	None
Testicular Extract				
Normal	17	14	3	0
*Adrenalectomized 8 to 18 days before ..	17	2	2	13
5-Hydroxytryptamine				
Normal	17	15	2	0
Adrenalectomized 2 months before ..	5	1	3	1
*Adrenalectomized 8 to 18 days before ..	12	6	3	3

the same dose in the adrenalectomized rat treated for several days with an amount of cortisone that had no effect on permeability (see Table I).

Discussion

As far as I know, no substance other than 5-hydroxytryptamine initiates gross acute transitory oedema in such a minute dose and volume. Histamine, for example, which is often said to cause oedema, does not initiate a similar state in doses of less than 100 μg. The effect in the adrenalectomized rats with 5-hydroxytryptamine-induced oedema contrasts with those in which the oedema was induced by testicular extract. The reason for this difference is at present unknown.

References

Benditt, E. P., and Rowley, D. A. (1956). *Science*, **123**, 24.

Kelemen, E. (1956). *Acta med. hungarica*, **9**, 125.

—— Majoros, M., Ivanyi, J., and Kovacs, K. (1950). *Experientia*, **6**, 435.

Miles, A. A., and Wilhelm, D. L. (1955). *Brit. J. exp. Path.*, **36**, 71.

Brit. J. Pharmacol. (1957), **12**, 30.

SOME ASPECTS OF THE PHARMACOLOGY OF AN HOMOLOGOUS SERIES OF CHOLINE ESTERS OF FATTY ACIDS

BY

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From the Department of Pharmacology, University of Birmingham

WITH AN APPENDIX BY ZU-YOONG KYI AND W. WILSON

(RECEIVED AUGUST 2, 1956)

The pharmacological effects of a series of fatty acid-choline esters have been studied on the isolated rabbit heart, the isolated guinea-pig ileum and the rat stomach. The effect changed with increasing chain length, and three different types of response were observed. The short-chain compounds produced depression of the isolated rabbit heart and spasm of the isolated guinea-pig ileum. Only one, butyrylcholine, had an erratic stimulating effect on hydrochloric acid secretion by the partially vagotomized rat stomach. Medium-chain compounds had a stimulating effect on the isolated rabbit heart, a mixed spasmogenic and relaxant effect on the isolated guinea-pig ileum, and no effect on the rat stomach. Long-chain compounds blocked the effect of acetylcholine on the isolated rabbit heart and the isolated guinea-pig ileum; they also depressed spontaneous hydrochloric acid secretion by the rat stomach. The nature of these three types of response is discussed.

The present investigation is concerned with the pharmacology of the choline esters of an homologous series of saturated straight-chain monobasic fatty acids.

Previous work was mainly concerned with the effect of these compounds on blood pressure. Hunt and Taveau (1911), in their extensive investigation of the pharmacology of a large number of choline derivatives, included the esters from acetylcholine (ACh) to pentanoylcholine and in addition palmitoylcholine, all of which occur in the series used in this work. They found that with increasing chain length the depressor effect declined, and a pressor effect was observed which in some cases became manifest after atropine but in others without atropine. These findings were confirmed and elaborated by le Heux (1921), Simonart (1932), Chang and Gaddum (1933), and Bovet and Bovet-Nitti (1948).

Fourneau and Page (1914) synthesized a series of these esters with even numbers of carbon atoms in the chain. Haemolytic action was most pronounced with the palmitic and stearic acid esters; it diminished with decreasing chain length, and was no longer found with members below dodecanoylcholine. In the present investigation an almost complete series of these esters was available. The effect of the chain length upon the pharmacological action was examined on the isolated mammalian heart, the motility of the small intestine, and the secretory response of the stomach.

METHODS

Isolated Rabbit Heart.—The usual Langendorff type of preparation with retrograde aortic perfusion was used. The composition of the perfusion solution used was: NaCl 9 g., KCl 0.42 g., $CaCl_2$ 0.24 g., $NaHCO_3$ 0.5 g., dextrose 1.0 g., distilled water 1 litre. The compounds tested were injected through the rubber-capped side-arm of the perfusion apparatus.

Isolated Guinea-pig Ileum.—The ability of some of the compounds to induce spasm was expressed in terms of the concentration which caused an effect equal to that produced by $10^{-6.5}$ M-ACh. This was determined upon the isolated guinea-pig ileum suspended in the 2.0 ml. bath of an automatic assay apparatus, similar to that described by Boura, Mongar, and Schild (1954). A 2 min. cycle was employed. The lever used was constructed according to the dimensions given by Schild (1947), and was not allowed to excurse more than 30° from the horizontal. This ensured an approximately linear relationship between the recorded height of contraction and the actual shortening of the muscle. Three preselected concentrations of the test spasmogen and the fixed concentration of ACh were set up in the apparatus

together. ACh was added repeatedly until a steady response had been obtained, when the remaining bottles of test spasmogen were switched in. Six contractions to each concentration of the test spasmogen and six to the standard spasmogen were obtained, the doses being added in random fashion. At the end of the cycle, a second test spasmogen was assayed in a similar manner. The heights of all contractions were measured in mm. Mean responses were computed, and those for the three concentrations of test spasmogen were plotted as ordinates against the negative logarithms of the corresponding molar concentrations as abscissae, and a curve drawn through these three points. The activity ratio for the test spasmogen was then determined graphically by dropping a perpendicular from the point on the curve corresponding to the mean response to ACh. The intersection of this line with the abscissa represented the ACh equivalent for the test spasmogen. Two determinations were carried out for each spasmogen using ileum from two guinea-pigs, and the means calculated. The results from the two experiments were in good agreement.

Hexamethonium iodide (HMI) was used to determine whether the lower members of the series produced spasm of the intestinal muscle by a ganglionic ("nicotinic") or a direct ("muscarinic") type of action. Equilibrium was attained with the standard spasmogen (ACh 0.05 μg./ml.) and then a single response to the test spasmogen was obtained. The tissue was then bathed in Tyrode solution containing 3.0 μg. HMI/ml. for exactly 2 min. The spasmogens were again added, and the effect of HMI on the response determined.

pA_2 values (Schild, 1947) for the esters against ACh were determined after 10 min. contact between the tissue and the antagonist. Percentage effects were calculated with respect to the difference between the response to the single and to the double dose of ACh, and not as % of the maximum. The tissue was always allowed to recover from the effect of the antagonist before proceeding with the experiment. Where this did not occur, as for example with the higher esters, fresh ileum from the same animal was used. Four determinations of pA_2 were made for each ester, using ileum from different guinea-pigs.

Errors in the quantitative assessments of activity due to adsorption of the esters on to the glassware (Marshall, 1955) were avoided by immersion of all glass apparatus in 33% HNO_3 overnight, followed by thorough washing in hot tap water and distilled water before subsequent use. No detergent was used.

In Vivo *Experiments on the Secretory Response of the Rat Stomach.*—White male rats weighing 200 to 250 g. were used. The abdomen was opened under ether anaesthesia and the pylorus exposed and ligated. A glass cannula carrying a short length of rubber drainage tubing was introduced through a small incision just orally to the ligature. Through this cannula the stomach was rinsed, after which the cannula was removed and a second ligature applied. The rats were then allowed to recover from the anaesthetic and left for 6 hr. At the end of this period the rats were anaesthetized once more, the total stomach contents measured, the *p*H determined with indicator papers, free HCl titrated with N/100 NaOH using methyl orange as indicator and total chlorides determined using Patterson's micro-technique (Harrison, 1943).

The esters were injected into the adductors of the thigh in aqueous solution. The control rats received a corresponding injection of tap water. In order to demonstrate stimulating effects, spontaneous secretion was reduced by partial vagotomy. The oesophagus was cut about 0.5 cm. above the cardiac sphincter between ligatures. A glass cannula was introduced through the opening pointing caudally into the cardiac end of the stomach. The stomach was rinsed through this cannula until the returning fluid was clear. Following this, 5 ml. of warm tap water was left in the stomach. By this means, the anterior vagus was cut, the posterior vagus damaged, and the rat stomach secreted small amounts of free HCl over a period of 2 hr. It responded to such secretory stimuli as histamine, neostigmine, and carbachol.

In all instances the iodides of the esters were administered, except for palmitoylcholine, which was used as the more soluble bromide.

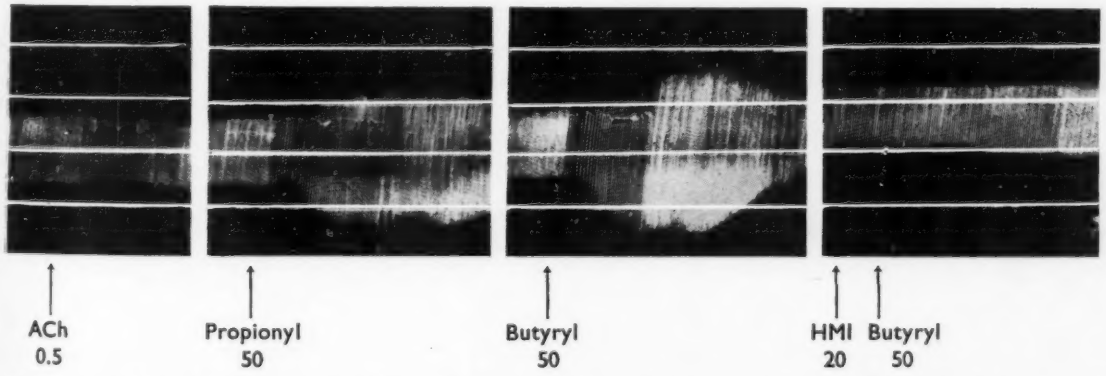


FIG. 1.—Isolated rabbit heart. (Langendorff preparation.) Records of ventricular contractions. Effect of acetyl-, propionyl-, and butyrylcholine. The action of butyrylcholine is greatly reduced by hexamethonium (HMI). Doses in μg.

Results

Effect on the Isolated Rabbit Heart

Three different types of effect were observed. The cardiac slowing and reduction of systolic contraction which characteristically occur with ACh were produced by the esters up to heptanoylcholine, although very much larger doses (50 μg.) had to be employed (Fig. 1).

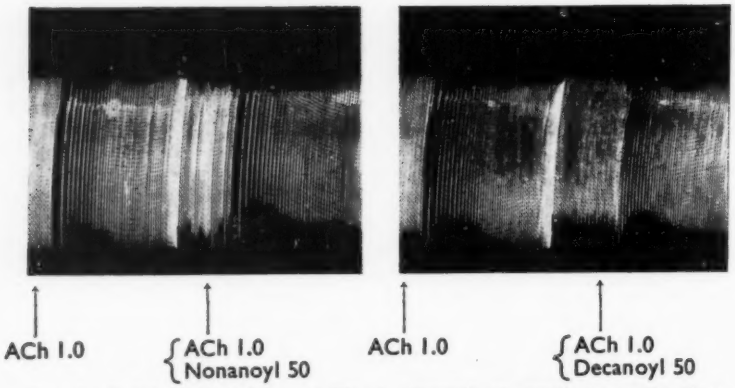
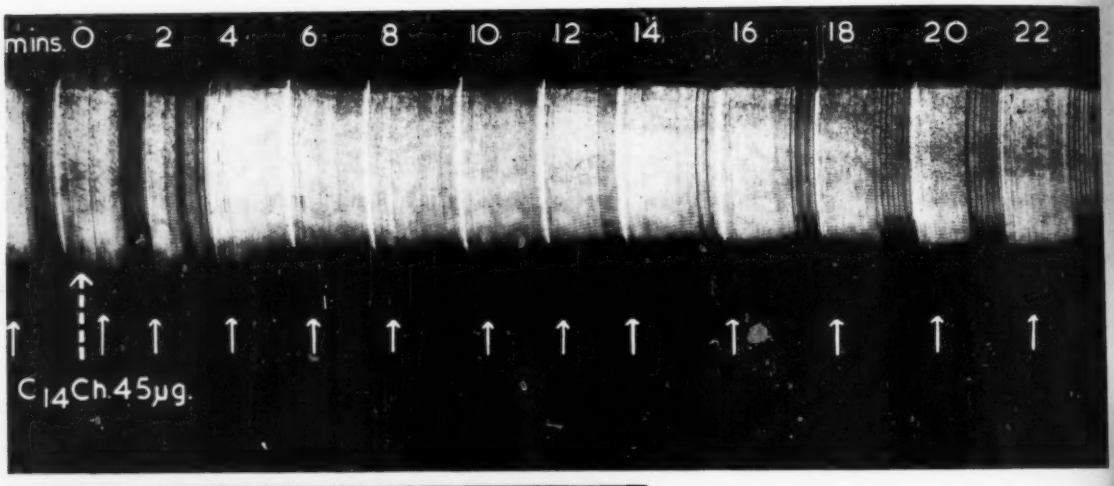


FIG. 2.—Isolated rabbit heart as Fig. 1. Records of ventricular contractions. Effect of nonanoylcholine and decanoylcholine on the response to acetylcholine (ACh). Amo unts expressed in μg.

With propionylcholine a stimulating effect made its appearance immediately following the depressant effect. This stimulation, which affected both the rate and height of contraction, was maximal with butyrylcholine, but was weaker with the pentanoyl and hexanoyl compounds and was not produced by the heptanoyl compound. When a dose of 20 μg. of hexamethonium was given immediately before the dose of ester, the stimulating effect of the propionyl compound was abolished, and that of the butyryl



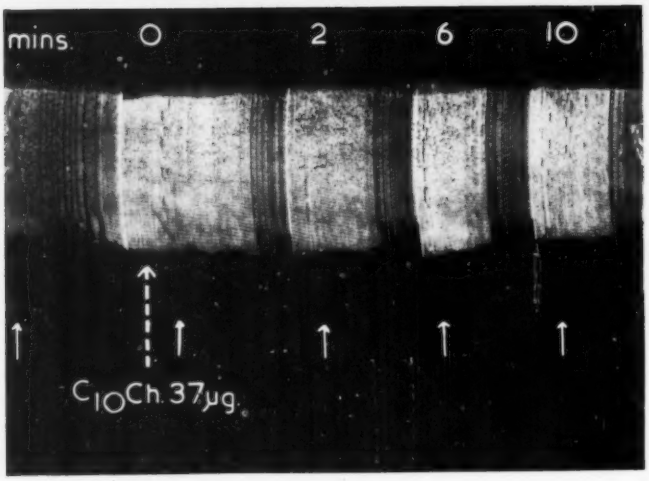


FIG. 3.—Isolated rabbit heart as Fig. 1. Records of ventricular contractions. Comparison of the ACh blocking effect of tetradecanoylcholine (C_{14}Ch) and decanoylcholine (C_{10}Ch). Decanoylcholine was given in an amount equimolar with the threshold dose of tetradecanoylcholine. At each small arrow 20 μg. ACh was given.

and pentanoyl esters was reduced by 70% and 85% respectively (Fig. 1).

The octanoyl and nonanoyl compounds seemed to be without effect on the isolated rabbit heart. When the chain length was increased further, however, a new phenomenon occurred which was first observed with the decanoyl compound (Fig. 2). This ester had a transitory blocking effect on the response to ACh when administered simultaneously. With a further increase in chain length the effect became more pronounced. Frequently it appeared after a short latent period and usually reached its maximum after 4 to 20 min. An assessment of the relative potency of these long-chain esters was made difficult by the persistence of their ACh blocking effect, which, in some instances, lasted for several hours. Occasionally the original response to ACh could no longer be obtained at all.

The effect of the esters was therefore compared by determining the threshold value for stearoylcholine and tetradecanoylcholine and comparing the effect of these compounds with that of the adjacent members used in corresponding amounts. Under such conditions stearoylcholine, palmitoylcholine, tetradecanoylcholine, and dodecanoylcholine produced the same effect. Decanoylcholine, however, was distinctly less potent in depressing the ACh response than any of the longer-chain esters (Fig. 3).

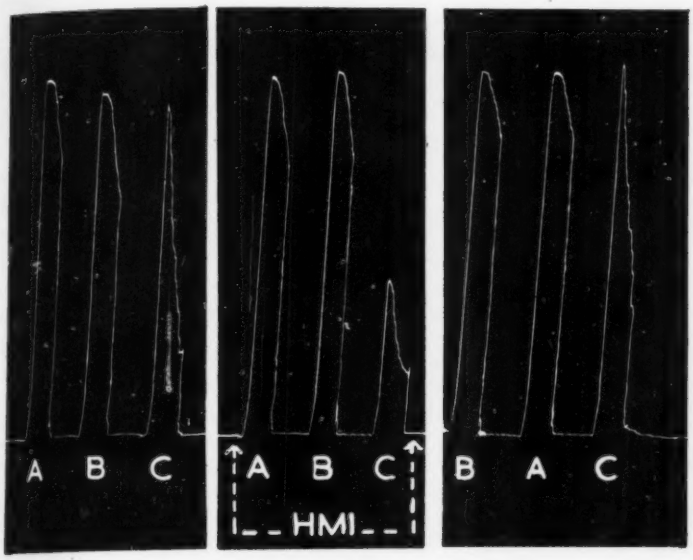


FIG. 4.—Isolated guinea-pig ileum. Hand-operated organ bath, 10 ml. capacity. Effect of hexamethonium (HMI) 3·0 μg./ml. on the response to 0·07 μg./ml. of ACh(A), 2·5 μg./ml. of propionylcholine (B) and 125 μg./ml. of butyrylcholine (C).

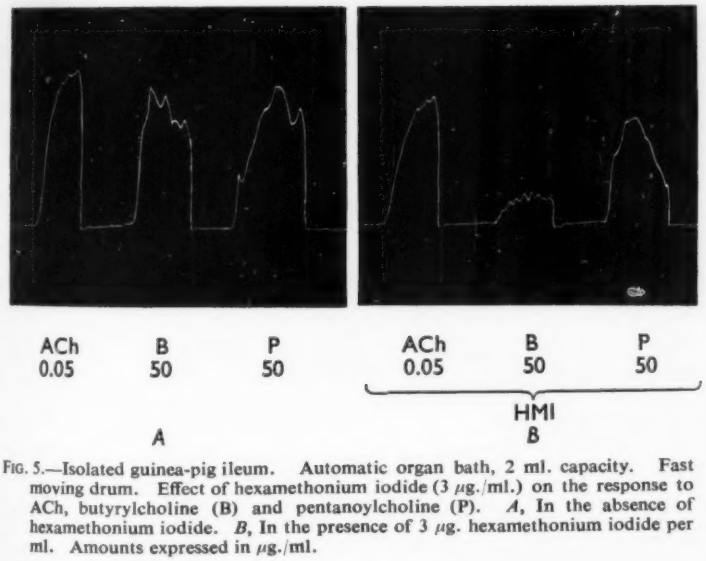


FIG. 5.—Isolated guinea-pig ileum. Automatic organ bath, 2 ml. capacity. Fast moving drum. Effect of hexamethonium iodide (3 μg./ml.) on the response to ACh, butyrylcholine (B) and pentanoylcholine (P). *A*, In the absence of hexamethonium iodide. *B*, In the presence of 3 μg. hexamethonium iodide per ml. Amounts expressed in μg./ml.

Effect on the Isolated Guinea-pig Ileum

Spasmogenic Activity.—The following molar concentrations determined in duplicate were equiactive with ACh $10^{-6.5}$ M (0.05 μg./ml.): propionylcholine $10^{-4.7}$, $10^{-4.6}$; butyrylcholine $10^{-3.6}$, $10^{-3.6}$. A rapid fall in spasmogenic activity occurred with increasing chain length. The mode of action changed in the transition from propionyl to pentanoylcholine. After a 2 min. exposure to 3.0 μg./ml. of HMI, the responses to ACh and propionylcholine were unaffected (Fig. 4). The responses to both butyryl and pentanoylcholine, however, were considerably affected by the same concentration of HMI (Fig. 5).

The responses to pentanoylcholine were less affected by HMI than those to butyrylcholine (Fig. 5); in the same experiment the ACh responses were also partially inhibited by HMI. However, this did not always occur, and there was considerable variation in the effect of HMI on the responses to ACh with different samples of tissue. In some experiments, using a hand-

operated organ bath, the effect of HMI 3.0 μg./ml. on the response of the isolated guinea-pig ileum to ACh varied from 5 to 10% inhibition to 3 or 4% potentiation. Of the spasmogens tested with HMI in the present work, butyrylcholine appeared to be most sensitive (Figs. 4 and 5). Both this compound and pentanoylcholine, in distinction to ACh and propionylcholine, produced contractions of the guinea-pig ileum which were characterized by a rapid increase of tone, followed by a phase during which the tonus fluctuated and gradually fell (Fig. 5). HMI, besides diminishing the height of these contractions, also inhibited the fluctuations of tone; with pentanoylcholine the fluctuations were converted to a pure relaxation. Barium has been observed to produced contractions of the guinea-pig ileum which resemble those described above, and this substance was stated by Feldberg (1951) to promote contraction partly by a ganglionic type of action. In order to obtain more

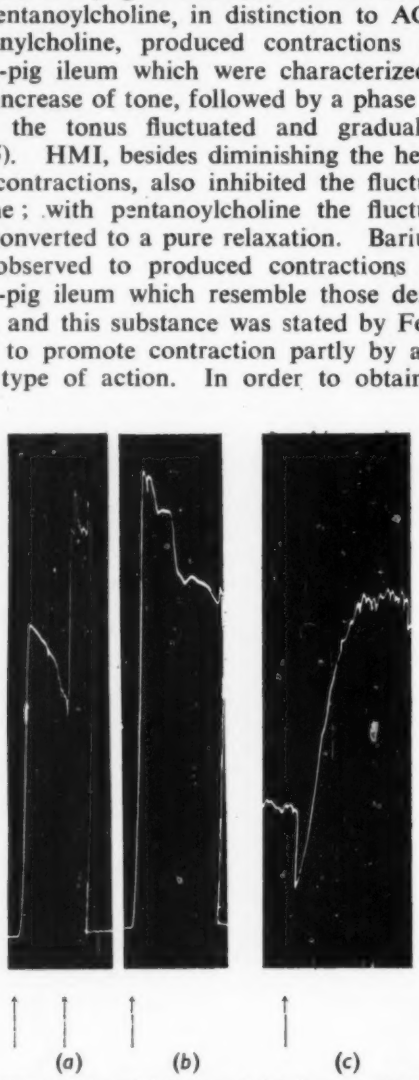


FIG. 6.—Effect of butyrylcholine on isolated guinea-pig ileum and rabbit duodenum. (*a*) Guinea-pig ileum. Organ bath 20 ml. capacity. At arrows, 500 μg. of butyrylcholine. (*b*) Guinea-pig ileum. Organ bath 20 ml. capacity. Effect of 10 μg. of neostigmine methylsulphate administered immediately before 250 μg. of butyrylcholine at arrow. (*c*) Rabbit duodenum. Organ bath 50 ml. capacity. At arrow, 250 μg. of butyrylcholine.

information regarding this complex response to butyrylcholine, further experiments were carried out with this substance.

Three possible explanations for the secondary relaxation came to mind. First, such relaxation might have been due to a non-specific depression of the muscle caused by the substance itself. Such a possibility, however, could be excluded by the finding that a subsequent administration of the same amount of butyrylcholine during the stage of relaxation still produced a spasmogenic effect (Fig. 6a). Secondly, relaxation might have been due to rapid hydrolysis of butyrylcholine by pseudocholinesterase, to the action of which it has been shown to be sensitive (Bayliss and Todrick, 1955). Such a possibility is excluded by the finding that secondary relaxation is not abolished by neostigmine methyl sulphate (Fig. 6b). In the third place the complex response to butyrylcholine might be due to a combined stimulation of both parasympathetic and sympathetic ganglia. This was borne out by an experiment on a piece of rabbit duodenum on which the relaxant component of the butyrylcholine response preceded the spasmogenic one (Fig. 6c).

Antagonism to ACh.—pA_2 values for the saturated fatty acid esters of choline from heptanoylcholine to stearoylcholine are recorded in Table I. A peak of anti-ACh activity was observed

TABLE I

THE pA_2 VALUES OF SOME FATTY ACID ESTERS OF CHOLINE, MEASURED AGAINST ACh ON THE ISOLATED GUINEA-PIG ILEUM

All values are the means of four determinations: standard deviations in parentheses. All of the compounds were iodides, except the palmitoyl ester which was used as the bromide.

$R—CO.O.CH_2.CH_2.\overset{+}{N}(CH_3)_3\ X^-$

Compound	R	pA_2
Heptanoylcholine	$-C_6H_{13}$	4·74 (0·17)
Octanoylcholine	$-C_7H_{15}$	4·69 (0·01)
Nonanoylcholine	$-C_8H_{17}$	4·97 (0·11)
Decanoylcholine	$-C_9H_{19}$	5·52 (0·08)
Undecanoylcholine ..	$-C_{10}H_{21}$	5·99 (0·15)
Dodecanoylcholine ..	$-C_{11}H_{23}$	6·46 (0·06)
Tetradecanoylcholine ..	$-C_{13}H_{27}$	6·79 (0·20)
Palmitoylcholine	$-C_{15}H_{31}$	6·37 (0·13)
Stearoylcholine	$-C_{17}H_{35}$	6·16 (0·21)

with tetradecanoylcholine. Heptanoylcholine and octanoylcholine were approximately equiactive as antagonists of ACh on the ileum. From octanoylcholine to dodecanoylcholine there was a steady rise in activity; the mean increase for each $-CH_2-$ increment in the carbon chain over this range of the series was 0.44 *p*A units. From tetradecanoylcholine to stearoylcholine the mean decrease in pA_2 for each $(-CH_2-)_2$ increment in the carbon chain was 0.32 *p*A units. Thus, the increase in activity up to tetradecanoylcholine took place at a greater "rate" than the decrease in activity above tetradecanoylcholine.

Effect on Gastric HCl Secretion of Rats

Fig. 7 shows the effect of the choline esters of fatty acids with even numbers of carbon atoms on the spontaneous gastric secretion of the rat. The lower members of this series are readily soluble, and large doses (165 mg./kg.) were used. The first three members, butyryl-, hexanoyl-, and octanoylcholine, did not produce any demonstrable effect. The results were all within the range of the controls. Although the mean of the results obtained with butyrylcholine (0.53 m.equiv) was somewhat higher than that of the controls (0.456 m.equiv), this difference was not significant. The solubility of the choline esters decreases with increasing chain length, and the maximum concentration obtainable with palmitoylcholine was only 3.3 g./l. The amount administered was therefore reduced to 33 mg./kg., and was used even for the more soluble compounds for the sake of uniformity. All the higher members of this series, starting with dodecanoylcholine, produced a marked inhibition of HCl secretion, although with decanoylcholine only the larger dose was effective. The *P* values were calculated according to White's modification of Wilcoxon's ranking method (White, 1952). The *P* value for all esters up to palmitoylcholine was below 0.001 (Table II). Significant inhibition was still obtained with the stearoyl ester ($P=0.05$).

TABLE II

INHIBITORY EFFECT OF THE LONGER-CHAIN FATTY ACID-CHOLINE ESTERS ON THE 6 HR. SPONTANEOUS SECRETION OF FREE HCl BY THE RAT STOMACH

Ester	Dose (mg./kg.)	No. of Animals Used	Mean Values in m.equiv.	*P*
Control	—	12	0·465	
Decanoyl	165	6	0·0115	0·001
Dodecanoyl ..	33	5	0·034	0·001
Tetradecanoyl- ..	33	6	0·132	0·001
Palmitoyl	33	8	0·085	0·001
Stearoyl	33	4	0·208	0·05

As the experiments on the heart and ileum had shown that the lower esters have a parasympathomimetic effect, it seemed possible that they might stimulate gastric secretion. When rats in which the spontaneous secretion had been reduced by partial vagotomy were injected with the shorter-chain esters, the results shown in Fig. 8 were obtained. Secretion was significantly raised with butyrylcholine, but remained unchanged when hexanoylcholine or octanoylcholine were used.

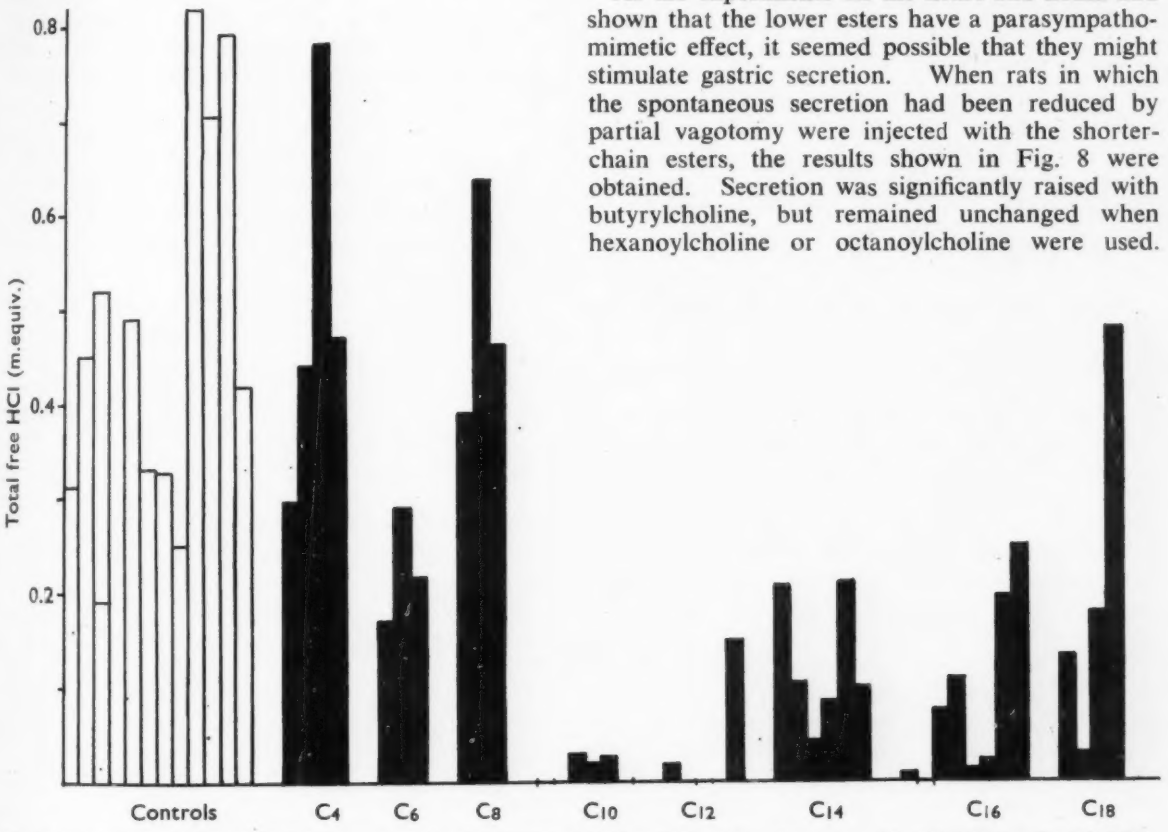


FIG. 7.—The effect of fatty acid-choline esters on the spontaneous gastric secretion of conscious rats. Ligated pylorus. Duration 6 hr. Each column represents the total free HCl secreted by one rat. Black columns represent the response after the intramuscular injection of the fatty acid-choline esters from butyrylcholine (C_4) to stearoylcholine (C_{18}). Blank columns represent the response after a corresponding injection of tap water. Compounds C_4 to C_{10} were given in doses of 165 mg./kg. and compounds C_{12} to C_{18} in doses of 33 mg./kg.

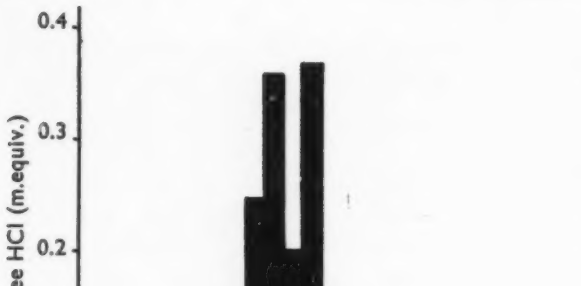


FIG. 8.—The effect of fatty acid-choline esters on the spontaneous gastric secretion of conscious rats. Ligated pylorus. Partial vagotomy. Distension with water. Duration 2 hr. Each column represents the total free HCl secreted by one rat. Black columns represent the response after the intramuscular injection of 165 mg./kg. of the fatty acid-choline esters from butyrylcholine (C_4) to octanoylcholine (C_8). Blank columns represent the response after a corresponding injection of tap water.

DISCUSSION

Three different types of effect of the fatty acid-choline esters could be demonstrated in the present investigation. With increasing chain length the muscarinic effect of ACh was replaced by a ganglionic effect and eventually by an ACh blocking action.

Muscarinic Effect.—The rapid decline in muscarinic action beyond ACh is well known and has been demonstrated as a waning depressor effect on blood pressure by Hunt and Taveau (1911), Chang and Gaddum (1933), and Bovet and Bovet-Nitti (1948), and as a decrease of the spasmogenic effect on isolated intestine by le Heux (1921) and by Chang and Gaddum (1933). In the present work on the isolated intestine and on the isolated heart, there was a steep decline in the depressant effect on rate and amplitude of contraction in the series of esters studied.

It has generally been assumed that the spasmogenic effect of ACh on the intestine, as well as the depressant effect on blood pressure and on the heart, is due to a direct action on parasympathetically innervated end-organs. In the case of the isolated heart, this view has recently been challenged by Perry and Talesnik (1953), who found that large doses (6 mg.) of hexamethonium abolished the depressant effect of ACh; they therefore concluded that ACh acted on intracardiac parasympathetic ganglia. Recent work by Douglas and Gray (1953) and Evans and Schild (1953), however, has shown that HMI has an effect on ganglion-free preparations, so that such a conclusion does not necessarily follow.

With further increase in chain length the muscarinic effect decreases still further. Hunt and Taveau (1911), Simonart (1932) and Bovet and Bovet-Nitti (1948) reported a slight depressor effect with butyrylcholine; Chang and Gaddum (1933) found "no real depressor effect" with this ester, but observed a spasmogenic effect on the isolated rabbit intestine. In the present work a slight depressant effect on the heart could be found occasionally with the esters from butyrylcholine to heptanoylcholine and a spasmogenic effect with butyrylcholine on the isolated guinea-pig ileum, though it is unlikely that these effects are due to a muscarinic mechanism.

Ganglionic Effect.—The pressor effect which is produced by ACh after atropine has been attributed to a stimulating effect on sympathetic ganglia leading to a release of sympathomimetic amines from adrenergic nerve endings and from the adrenal medulla. A similar response with some of the higher esters after atropine was found by Hunt and Taveau (1911), Chang and Gaddum (1933), and Simonart (1932). The last author claimed that this effect increased with increasing chain length from ACh to butyrylcholine. Bovet and Bovet-Nitti (1948) found a pressor effect without previous atropinization with the esters pentanoylcholine, hexanoylcholine and octanoylcholine.

The present experiments were carried out in the absence of atropine. On the isolated heart, the first appearance of an effect which was the reverse of that produced by ACh occurred with propionylcholine and consisted in an increase in rate and extent of contraction. With increasing chain length, this effect increased further, reaching its maximum with butyrylcholine, waned again with pentanoylcholine and still further with hexanoylcholine, and could no longer be detected with heptanoylcholine. As this response could be effectively blocked with HMI, stimulation of intracardiac sympathetic ganglia seemed the most likely explanation.

A stimulating action on the isolated rabbit or cat heart by ganglionic stimulants such as nicotine and ACh after atropine was described by Hoffmann, Hoffmann, Middleton and Talesnik (1945) and more recently on the isolated rabbit auricles by Kottegoda (1953) and Ginzel and Kottegoda (1953). The latter authors found that this effect could be blocked by HMI, and assumed

the presence of peripheral sympathetic ganglia within the auricular tissue.

On the isolated guinea-pig ileum, the first evidence suggestive of a ganglionic component of the response occurred with butyrylcholine. Although this compound, as well as pentanoylcholine, still produced a spasmogenic effect, the response differed in several aspects from that produced by ACh and propionylcholine. Whereas the response to the two latter esters was little influenced by HMI, this ganglionic blocking agent almost completely abolished the response to butyrylcholine, which suggested that the spasmogenic effect in this case was exerted on the intramural parasympathetic ganglia. The experimental evidence illustrated in Fig. 6 suggested that the stimulation of sympathetic intramural ganglia was responsible for the spasmolytic component of the response to butyrylcholine. The existence of such ganglia has been suggested by Ambache (1951), who demonstrated the reversed action of nicotine on the isolated mammalian intestine after cholinergic paralysis with *Clostridium botulinum* toxin. HMI, given in an amount that almost abolished the spasmogenic effect of butyrylcholine (3.0 μg./ml.), did not appear to affect this rapid relaxation, so that the sympathetic ganglia involved would appear more resistant to HMI than the parasympathetic ones. Only a small effect of this sort was produced on the response to pentanoylcholine, which seemed to point to a mainly non-ganglionic mechanism. The response to this ester, however, was erratic and in many instances no spasmogenic effect could be found.

ACh Blocking Effect.—The ACh blocking effect of these fatty acid-choline esters has not previously been described, though such an effect has been reported with other choline esters such as the benzilic acid ester by Ing, Dawes and Wajda (1945), dibutoline by Swan and White (1944), and benzoyl choline by Akcasu, Sinha and West (1952).

The blocking effect appeared in all three test preparations used. On the isolated heart and on the rat stomach the first member of the series to show the effect was decanoylcholine; heptanoylcholine had considerable activity on the isolated guinea-pig ileum. The only preparation on which accurate quantitative assessment could be carried out was the isolated guinea-pig ileum. There was a well-marked and progressive increase of anti-ACh activity from octanoylcholine to tetradecanoylcholine and a decrease from tetradecanoylcholine to stearoylcholine.

It seems possible that this phenomenon is associated with a gradation in physical properties, as the series is ascended. It will be seen that from nonanoylcholine to tetradecanoylcholine the differences in pA_2 values between adjacent homologues gradually fall (0.55 between nonanoylcholine and decanoylcholine, 0.45 between decanoylcholine and undecanoylcholine and between undecanoylcholine and dodecanoylcholine, 0.33 between dodecanoylcholine and tetradecanoylcholine). A similar phenomenon is also seen when the physical or physico-chemical properties of other homologous series are considered, such as the boiling points of the paraffins and the melting and boiling points of the monobasic carboxylic acids. No supplies of the tridecanoyl ester were available, hence the exact position of peak atropine-like activity could not be ascertained.

We have much pleasure in thanking Professor A. C. Frazer and Professor J. H. Burn for their advice and criticism, and our technical assistants, mainly Mrs. H. Bishop, Mr. A. G. Whybourn, and Mr. B. D. Williams, for their help. We also wish to thank Colonial Products Council for financial assistance. The present work was carried out while one of us (A. R. T.) was holding a scholarship of this body.

References

Akcasu, A., Sinha, Y. K., and West, G. B. (1952). *Brit. J. Pharmacol.*, **7**, 331.
Ambache, N. (1951). Ibid., **6**, 51.
Bayliss, B. J., and Todrick, A. (1955). *Biochem. J.*, **62**, 62.
Boura, A., Mongar, J. L., and Schild, H. O. (1954). *Brit. J. Pharmacol.*, **9**, 24.
Bovet, D., and Bovet-Nitti, F. (1948). *Structure et activité pharmacodynamique des médicaments du système nerveux végétatif; Adrénaline, acétylcholine, histamine, et leurs antagonistes.* Basel: Karger.
Chang, H. C., and Gaddum, J. H. (1933). *J. Physiol.*, **79**, 255.
Douglas, W. W., and Gray, J. A. B. (1953). Ibid., **119**, 118.
Evans, D. H. L., and Schild, H. O. (1953). Ibid., **122**, 63P.
Feldberg, W. (1951). Ibid., **113**, 483.
Ginzel, K. H., and Kottegoda, S. R. (1953). *Brit. J. Pharmacol.*, **8**, 348.
Harrison, G. A. (1943). *Chemical Methods in Clinical Medicine*, 2nd ed., p. 442. London: Churchill.
Heux, J. W. le (1921). *Pflüg. Arch. ges. Physiol.*, **189-192**, 280.
Hoffmann, F., Hoffmann, E. J., Middleton, S., and Talesnik, J. (1945). *Amer. J. Physiol.*, **144**, 189.
Hunt, R., and Taveau, R. de M. (1911). *Hyg. Lab. Bull.*, **73**, Treasury Dept.
Ing, H. R., Dawes, G. S., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 85.
Kottegoda, S. R. (1953). *Brit. J. Pharmacol.*, **8**, 83.
Marshall, P. B. (1955). Ibid., **10**, 270.

Perry, W. L. M., and Talesnik, J. (1953). *J. Physiol.*, **119**, 455.
Schild, H. O. (1947). *Brit. J. Pharmacol.*, **2**, 189.
Simonart, A. (1932). *J. Pharmacol.*, **46**, 157.
Swan, K. C., and White, N. G. (1944). Ibid., **80**, 285.
White, C. (1952). *Biometrics*, **8**, 33.

APPENDIX

Preparation of the Choline Esters of Fatty Acids

By Zu-Yoong Kyi and W. Wilson

Several of the choline ester salts made during the present investigation were described by Fourneau and Page (1914), who failed to state the melting points of the iodides. Loury (1939) outlined a more convenient method of preparation for choline ester salts, but gave no details. In the present work, Loury's method was used, as follows:

$$R.CO.Cl + HO.CH_2.CH_2.N(CH_3)_2 \longrightarrow R.CO.O.CH_2.CH_2.N(CH_3)_2 \xrightarrow{CH_3I} R.CO.O.CH_2.CH_2.\overset{+}{N}(CH_3)_3\ I^-$$

The products so obtained are listed in Table III. The iodides of the higher members were only slightly soluble in water, and it was necessary to make the corresponding bromides. This was easily accomplished by using methyl bromide instead of methyl iodide in the above reaction scheme. Abderhalden, Paffrath, and Sickel (1925) quote m.p. 72° C. (for palmitoylcholine bromide, which is substantially lower than the m.p. 185° C. observed by us. However, previous workers have observed polymorphism in choline ester salts (Fourneau and Page, 1914; Loury, 1939). These salts decompose slightly above their melting points, and trimethylamine salts are formed:

$$R.CO.O.CH_2.CH_2.\overset{+}{N}(CH_3)_3\ I^- \longrightarrow R.CO.O.CH:CH_2 + N(CH_3)_3,HI$$

Experimental. — Iodides: 2-Dimethylaminoethanol (1 mole) was added slowly to the appropriate acid chloride (1.1 moles) (prepared by the method of Bauer, 1946), dissolved in dry benzene (5 to 10 moles), with cooling to 0° C. After 24 hr. water was added and the organic solvent layer removed. With the higher members, it was necessary to add alcohol as well as water to dissolve the solids. Aqueous NaOH was added to the aqueous layer, and the basic ester isolated by chloroform or ether extraction. The ester was dissolved in ethanol or *iso*propanol and refluxed with methyl iodide. For the first three members of the series, methyl iodide was added to the crude chloroform extract. The products are listed in Table III.

Bromides: The basic esters, prepared as already described, were boiled with a solution of methyl bromide (approximately 15%) in chloroform; the solvent was distilled off, and the residue recrystallized.

Thermal Decomposition of Choline Ester Salts. —Dodecanoylcholine iodide (5 g.) was heated at 200 to 230° C. (bath temperature) for 30 min. Extraction of the black mass with hot ethanol gave trimethylammonium iodide (1 g., 44%). The same product was obtained from propionyl and palmitoylcholine iodides.

References

Abderhalden, E., Paffrath, H., and Sickel, H. (1925). *Pflüg. Arch. ges. Physiol.*, **207**, 241.
Bauer, S. T. (1946). *Oil and Soap*, **23**, 1 (see Wood, T. R., Jackson, F. L., Baldwin, A. R., and Longenecker, H. E. (1944). *J. Amer. chem. Soc.*, **66**, 287).
Fourneau, E., and Page, H. J. (1914). *Bull. Soc. chim. France*, **15**, 544.
Loury, M. (1939). *C.R. Acad. Sci., Paris*, **209**, 682.

Table III

Choline Ester Iodides. $R.O.CH_2.CH_2.\overset{+}{N}(CH_3)_3\ I^-$

Name	Recrystallized from	Yield %	m.p. °C.	Formula	% Found				% Calculated			
					C	H	N	I	C	H	N	I
Propionyl ..	Ethanol	32	130·5–132	$C_8H_{18}O_2NI$	33·7	6·3		43·9	33·45	6·3		44·25
Butyryl	*iso*Propanol	77	93–94	$C_9H_{20}O_2NI$	36·1	6·6		42·3	35·9	6·6		42·2
Pentanoyl ..	Ethanol	69	107–108	$C_{10}H_{22}O_2NI$	38·5	6·9		40·45	38·1	7·0		40·3
Hexanoyl ..	,,	76	125–126	$C_{11}H_{24}O_2NI$	40·3	7·2		38·3	40·1	7·3		38·6
Heptanoyl ..	,,	40	137–138	$C_{12}H_{26}O_2NI$	42·3	7·4		36·9	42·0	7·6		37·0
Octanoyl ..	,,	59	146–147	$C_{13}H_{28}O_2NI$				35·6				35·6
Nonanoyl ..	,,	41	154·5–156	$C_{14}H_{30}O_2NI$	44·5	8·0		34·8	45·3	8·1		34·2
Decanoyl ..	Methanol	52·5	158–159	$C_{15}H_{32}O_2NI$	45·9	8·4		32·8	46·75	8·3		33·0
Undecanoyl ..	,,	66	164–165	$C_{16}H_{34}O_2NI$	48·2	8·4		31·9	48·1	8·5		31·8
Dodecanoyl ..	,,	77	164–166	$C_{17}H_{36}O_2NI$				30·6				30·75
Tetradecanoyl ..	,,	75	162–163	$C_{19}H_{40}O_2NI$	51·8	9·0			51·7	9·1		
Palmitoyl ..	,,	80	160–161	$C_{21}H_{44}O_2NI$			2·7	26·6			3·0	27·1
Palmitoyl *bromide*	Acetone-ethanol	86	185	$C_{21}H_{44}O_2NBr$								
Stearoyl ..	Methanol	77	161–162·5	$C_{23}H_{48}O_2NI$	55·3	9·4		25·0	55·5	9·7		25·55

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HISTAMINE RELEASE INHIBITION *IN VITRO* AND ANTIANAPHYLACTIC EFFECTS *IN VIVO* OF SOME CHEMICAL COMPOUNDS

BY

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Several different types of compounds inhibited the release of histamine from the " platelet fraction " of rabbit blood when antigen was added to the blood of a sensitized rabbit *in vitro*. These compounds were all very toxic *in vivo* and could be tested only at low levels for their ability to protect animals against anaphylaxis. None of the compounds gave significant protection to sensitized animals when antigen was given intravenously. Three compounds protected animals against antigen administered as an aerosol. None of these showed any promise of clinical usefulness.

Of a number of compounds which have been reported to alleviate allergic symptoms, or to protect against anaphylaxis, none prevented the release of histamine *in vitro* to any considerable extent.

The efficacy of the antihistamines has given considerable credence to the concept that the release of histamine from sensitized tissue cells is a major event in anaphylactic and allergic reactions. There is now wide acceptance of the view that the antigen–antibody reaction, by an unknown mechanism, causes severe injury to certain tissue cells ; histamine and perhaps other highly active compounds which are released from the injured cells produce most of the acute symptoms of many anaphylactic and allergic reactions.

The shortcomings of the antihistamines have encouraged workers in this field to think in terms of inhibiting the release of histamine (Spain and Strauss, 1951). Trethewie (1951, 1952) has reported that aspirin and *p*-aminosalicylic acid, which inhibit anaphylaxis but have no antihistaminic action, inhibit the release of histamine by antigen in the perfused lung from a sensitized guinea-pig. Trethewie and others (Coburn and Kapp, 1943 ; Campbell, 1948 ; Lepper, Caldwell, Smith, and Miller, 1950) suggested that the salicylates interfere with the reaction by preventing the union between antigen and antibody. Moss, Beiler, and Martin (1950) attempted to block the release of histamine by inhibiting histidine decarboxylase. Because of the abundance of preformed, loosely bound histamine in the tissues (McIntire, 1955a, 1955b), and because of the long "half-life" of tissue histamine (Schayer, 1952), this approach does not seem reasonable, unless a dearth of histamine could be effected by the long-term treatment with histidine decarboxylase inhibitors. Other workers (Malkiel and Werle, 1951) have not been able to confirm the findings of Moss and his colleagues on the inhibition of anaphylaxis by (+)-catechin.

In our laboratories a screening programme was begun in 1946 for chemicals which would inhibit the release of histamine from the " platelet fraction " when antigen was added to the blood from a sensitized rabbit *in vitro*. We were searching for compounds which might inhibit the reaction at any point before the release of histamine. It was hoped that some of the inhibitors found in this screening programme would inhibit anaphylaxis *in vivo* and be of some value for the treatment of allergic disorders. Approximately 2,500 compounds have been tested. This paper describes the results obtained with the most interesting compounds of this group.

METHODS

Details of rabbit sensitization and bleeding were presented in a previous publication (McIntire, Roth, and Richards, 1949). The blood was kept in an ice-bath until used. All glassware in which blood was handled was coated with silicone, either General Electric Dri Film 9987 or Dow Corning Pan Glaze. *In vitro* histamine release reactions were carried out

as follows: The chemical to be tested, in isotonic solution, was pipetted into a heavy duty 12 ml. conical centrifuge tube in an ice-bath. The volume was diluted to 2.6 ml. with isotonic sodium chloride. To this solution 2 ml. of blood was added, followed by thorough mixing, and then was added 1 ml. of a 1:100 dilution of egg white in saline. The contents of each tube were mixed thoroughly; the tubes were placed in a 37° C. bath for 20 min. and then returned to the ice-bath. Blood cells were removed by centrifugation at approximately 4° C. A 4.5 ml. aliquot of the supernatant fluid was taken for histamine determination (Code and McIntire, 1956). The final volume of histamine-release reaction-mixture was 5.6 ml., but, since 0.6 ml. of this was blood cells, drug concentrations and histamine assays were calculated for the 5 ml. of extracellular solution.

The inhibition of histamine release was calculated as follows:

$$\% \text{ inhibition} = \frac{H_c - H_i}{H_c - H_b} \times 100, \text{ where}$$

H_b histamine in plasma from "blank" reaction, without egg white

H_c histamine in plasma from reaction with egg white

H_i histamine in plasma from reaction with egg white in the presence of the chemical being tested for inhibition.

Frequently H_c would account for as much as 75% of the total histamine in the blood. H_b was rarely more than 20%, and usually less than 10%, of the total histamine. In most experiments H_b was not more than 20% of H_c.

Compounds were tested *in vivo* for antihistaminic activity in guinea-pigs by exposing the animals to a histamine aerosol 30 min. after intraperitoneal injection of the test drug. Antianaphylactic activity was tested in guinea-pigs which had been actively or passively sensitized to whole egg white. These were exposed to aerosolized antigen according to the method of Herxheimer (1952). In both tests, protection was evaluated by the prolongation of exposure before development of critical signs.

Irritation was tested by subcutaneous injection in rabbits, instillation of solutions into the rabbit eye, and, ultimately, testing solutions in the eyes of human volunteers. Administration of aerosols of the drugs to guinea-pigs provided another assessment of irritation to mucous membranes.

Results

Inhibitors of Histamine Release

Early in our screening programme it became evident that many types of compounds, if present at a concentration of 0.5 to 1 mg./ml., would inhibit histamine release in rabbit blood. Most of these compounds had no effect at a concentration of 0.1 mg./ml. or less. Subsequently, all compounds were screened at a concentration of 0.04 mg./ml. in the diluted blood. Only compounds producing an inhibition of at least 40% were considered for *in vivo* studies.

Table I lists those compounds of various structure which met the criteria regarding potency set forth above. It will be noted that most of them are of strongly cationic character; many are quaternary ammonium bases. Only one (compound B) is nitrogen free, but it is strongly cationic. Compounds A and C proved the most potent *in vitro*; they exerted a definite effect at a very low concentration, 0.00002 M. The maximal inhibition usually occurred at 0.0002 M. In one instance at the highest concentration (compound C), there was a slight to moderate increase, rather than a decrease of histamine release, which is designated by −20 in Table I.

Several compounds which had shown activity in the foregoing *in vitro* tests were selected for further evaluation. Each compound was first administered to groups of normal guinea-pigs. Thirty minutes later they were exposed to a histamine aerosol, of a concentration in which unprotected animals developed marked dyspnoeic symptoms within 2 min. Any compound which afforded significant protection to the animals was rejected, since antihistaminic activity would mask any evaluation of the ability of the compound to inhibit histamine release.

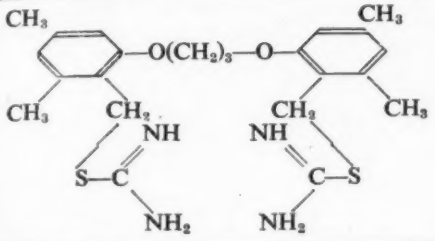
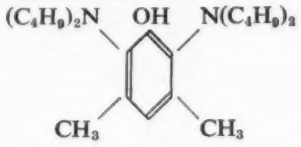
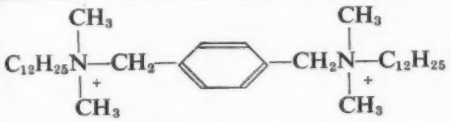
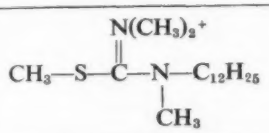
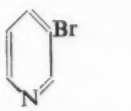
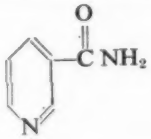
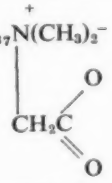
If little or no protection was evident, such compounds were then tested for antianaphylactic activity. Earlier tests involved intravenous challenge in actively or passively sensitized animals, but this was found to be too severe a test. Subsequent testing was by the Herxheimer method, in which sensitized animals were exposed to aerosol of egg white. Sixty-two control animals developed shock symptoms within an average of 2.2 min.

Of the compounds shown in Table I, A, E, and H were found to afford no protection against egg-white aerosol challenges at the maximal tolerated dose of the drug (20 mg./kg. for A and H, 10 mg./kg. for E). Compounds F and I were found to be too toxic to consider for testing.

Additional groups of guinea-pigs, or the same groups tested in cross-over experiments, were pretreated with one of the three remaining compounds, B, C, or D. These were the only compounds which had not been eliminated by previous *in vivo* screening tests on the basis of the limitations described. The protective dose was administered intraperitoneally 30 to 180 min. before exposure to the antigen aerosol. In doses of 10 mg./kg. compound C lengthened the shock time to an

TABLE I

INHIBITION OF HISTAMINE RELEASE BY DIFFERENT TYPES OF COMPOUNDS

Ref. Letter	Inhibitor	% Inhibition* at Various Concentrations			
		4×10^{-4}M	2×10^{-4}M	7×10^{-5}M	2×10^{-5}M
A		78	78	39	59
B	$C_{12}H_{25}S^{+}(CH_3)_2$	31	48	42	8
C		−20	61	70	41
D		79	88	45	16
E		2	47	24	0
F		42	45	20	0
G			51 ($1\cdot1\times10^{-4}$M)		
H		57	67	24	10
I	$C_{18}H_{37}N^{+}(CH_3)_3$		69 (9×10^{-5}M)		

*The values given for % inhibition are the means of experiments on four rabbit bloods. As the standard deviation was independent of the % inhibition, a pooled standard deviation was determined. The standard error of the mean of four rabbit bloods is 10.

average of more than 10 min., compound B to 8 min., and compound D to 9.4 min.

It should be emphasized that, after the control animals had been removed from the chamber upon signs of shock appearing, these signs usually progressed further, sometimes terminating in anaphylactic death. Likewise, if they were allowed to remain in the chamber, the same factors would inevitably cause anaphylactic death. After injection of compounds B and D, no progression of signs was noted when the animals were removed from the chamber. Therefore, two other groups were deliberately left in for the arbitrary period of 10 min. In these animals there was no progressive increase in the signs of shock, and all the animals survived the 10 min. exposure. The results are summarized in Table II.

TABLE II

DRUG PROTECTION AGAINST MICROSHOCK ANAPHYLAXIS

Drug*	No. of Animals	Dose		Shock Time, Minutes (Mean and Standard Error)†
		mg./kg.	% of LD50	
Controls	62	10	20	2·15 ± 0·23
C	16	10	20	> 10
B	41	10	20	8·07 ± 0·74
D	27	5	20	7·31 ± 0·92
	9	10	40	9·38 ± 0·98
B	5	10	20	10‡
D	5	10	40	10‡

* See Table I for formulae of compounds tested.

† The means and their standard errors were calculated using the one-sided censored normal distribution method given by A. Hald, *Statistical Methods with Engineering Applications*, and A. Hald, *Statistical Tables and Formulas* (London: Chapman & Hall, 1952).

‡ Deliberately left in 10 min. Signs did not progress further, and all animals survived.

The limiting factor in the above experiments was that the protective dose in these animals was also a toxic dose. In a few experiments where the dose was increased, the toxic effects of the drug were great enough to cause a higher mortality rate in the treated animals than in the controls.

Because of this limitation, topical application appeared to be the remaining possibility for clinical application. Tests in rabbit corneas showed compound C to be irritating upon local instillation. Compounds B and D, similarly tested, showed no corneal irritation or damage. However, when these were dropped into the eyes of human volunteers, both were judged to cause sufficient discomfort to preclude their acceptability for this use. Aerosols of compounds B and D caused marked sneezing, retching, and coughing in guinea-pigs exposed to mists for only a few minutes. This, and the observations on the human eye, were evidence against their suitability for use on mucous membranes. A limited trial in patients of compounds B and D was attempted by local application to the scarified area of scratch tests for specific antigens. Pretreatment with these compounds gave no evidence for a reduction of the local reaction produced by the antigen.

Other Compounds Giving Protection against Anaphylaxis

Several drugs have been reported to alleviate allergic symptoms or to protect experimental animals against anaphylaxis (Goth and Holman, 1947; Wolfsohn, 1944; Wilson and DeEds, 1948; Raiman, Later, and Necheles, 1947; Hiller, 1950). An attempt was made to test these preparations, including several of the antihistamines, for inhibition of histamine release. All were tested at 0.04 mg./ml., except aspirin and sodium salicylate, which were tested at concentrations of 0.2 mg./ml. The results are presented in Table III.

TABLE III

INHIBITION OF HISTAMINE RELEASE BY ANTIANAPHYLACTIC AND ANTIALLERGIC DRUGS

Compound	% Inhibition	Compound	% Inhibition
Methapyriline hydrochloride	31	Nicotinic acid	22
Tripelennamine ,,	5	Pentobarbitone sodium	0
Diphenhydramine ,,	25	Rutin	0
Pyrilamine maleate	6	Adenosine triphosphate	11
Chlorcyclizine hydrochloride	8	Theophylline	3
Procaine	11	(+)-Catechin	22
Atropine	0	Thorotrast	20
Sodium acetylsalicylate	0	Oleic acid	3
,, salicylate	0	Cortisone	7

DISCUSSION

The failure of aspirin to inhibit histamine release significantly *in vitro* is particularly interesting, inasmuch as we have confirmed (in unpublished work) other reports (Campbell, 1948; Lepper *et al.*, 1950) on the protection of rabbits against anaphylaxis by aspirin. To pursue this point we studied the histamine release in the blood of rabbits which had been treated with sufficient aspirin to protect them against anaphylaxis. Again aspirin failed to inhibit the release of histamine in rabbit blood *in vitro*. These results were somewhat inconsistent with the view that aspirin protected against anaphylaxis by interfering with antigen–antibody interaction.

Our results with oleic acid were not necessarily contradictory to those of Spain and Strauss (1951), who reported that this compound inhibits histamine release. They used approximately 10 times the concentration of oleic acid which we used. Their histamine release system employed human anti-ragweed serum, ragweed antigen, and normal rabbit blood, whereas we used egg white and blood from actively sensitized rabbits. The failure of

the other compounds of Table III to inhibit histamine release significantly does not mean that they might not protect the animal against anaphylaxis. It only suggests that their mode of protection is other than by the prevention of histamine release.

In a previous publication (McIntire, Roth, and Sproull, 1951), it was reported that a relatively slight change of chemical structure, such as the substitution of a carbomethoxy group for a carbamide group in the β-position of certain quaternary pyridinium compounds, changes an inhibitor of histamine release (compound G) into a histamine releaser. Histamine release from cells can be effected as the result of the antigen–antibody reaction, as well as by certain chemicals of relatively small molecular size, such as compounds of the structure referred to above. Thus, a compound only slightly different from a histamine releaser is able to prevent the release of histamine by either procedure. This suggests the possibility of a common basic mechanism of histamine release, and also of a common site of action of the inhibitor in preventing the release when the cells are exposed either to the antigen–antibody reaction or the specific chemical histamine release agent. The cell membrane is likely to be the site of these interactions.

The significant protection afforded by compounds B, C, and D against anaphylactic reactions in guinea-pigs is assumed to be due to some interference with histamine release, since none of them was effective against histamine aerosol shock in the doses employed. In those exposed to antigen aerosol, the interference with the progression of the signs would justify the assumption that the compounds apparently prevented the release of some, but not all, of the histamine resulting from the antigenic challenge.

We are indebted to the Misses M. Sproull, A. Hawthorne, and I. M. Shepperd, and Mr. B. B. Morphis, for technical assistance, and to Dr. S. M. Feinberg for the human scratch testing.

References

Campbell, B. (1948). *Science*, **108**, 478.
Coburn, A. F., and Kapp, E. M. (1943). *J. exp. Med.*, **77**, 173.
Code, C. F., and McIntire, F. C. (1956). *Methods of Biochemical Analysis*, 1st ed., vol. 3, pp. 85–90. New York: Interscience.
Goth, A., and Holman, J. (1947). *J. Pharmacol.*, **89**, 379.
Herxheimer, H. (1952). *J. Physiol.*, **117**, 251.
Hiller, E. (1950). *Münch. med. Wschr.*, **92**, 875.
Lepper, M. H., Caldwell, E. R., Smith, P. K., and Miller, B. F. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 254.
McIntire, F. C. (1955a). *Ciba Foundation, Symposium on Histamine*, p. 170. London: Churchill.
—— (1955b). Ibid., p. 416.
—— Roth, L. W., and Richards, R. K. (1949). *Amer. J. Physiol.*, **159**, 332.
—— —— and Sproull, M. (1951). Ibid., **167**, 233.
Malkiel, S., and Werle, M. D. (1951). *Science*, **114**, 98.
Moss, J. N., Beiler, J. M., and Martin, G. F. (1950). Ibid., **112**, 16.
Raiman, R. J., Later, E. R., and Necheles, H. (1947). Ibid., **106**, 368.
Schayer, R. W. (1952). *J. biol. Chem.*, **199**, 245.
Spain, U. C., and Strauss, M. B. (1951). *J. Allergy*, **22**, 47.
Trethewie, E. R. (1951). *Aust. J. exp. Biol. med. Sci.*, **29**, 443.
—— (1952). *Med. J. Aust.*, **1**, 638.
Wilson, R. H., and DeEds, F. (1948). *Science*, **107**, 369.
Wolfsohn, G. (1944). *Palest. Near East med. J.*, **3**, 11.

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EXPERIMENTS WITH THE TRYPANOCIDAL COMPOUND "528" IN WEST AFRICA

BY

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Studies have been made on the use of the chloride salt of "528" against cattle trypanosomiasis in Nigeria. Toxic effects, terminating in death, were produced in cattle receiving the drug at 5 mg./kg. and above. The maximum permissible dose for field use in Nigeria was found to be 2 mg./kg. The drug had an appreciable curative action against a syringe-transmitted strain of *T. congolense*, but had no curative effect against two strains of *T. vivax*. It is concluded that "528" would be of very limited value in the treatment of cattle in West Africa, where *T. vivax* is the more important cause of cattle trypanosomiasis.

The existence of activity against *Trypanosoma congolense* in cinnoline derivatives was first described by Keneford, Lourie, Morley, Simpson, Williamson, and Wright (1948). The synthesis of one of these compounds ("528"; N^1:N^3-bis(4′-aminocinnolyl-6′)-guanidine dimethiodide) was subsequently described by Lourie, Morley, Simpson, and Walker (1951). This substance had high activity against *T. congolense* infections in mice; it was approximately one half as toxic and one half as active as antrycide methylsulphate. It appeared from these preliminary observations that "528" was worthy of trial against cattle trypanosomiasis in Africa. The commonest species of trypanosome encountered in West African cattle is *T. vivax* (Unsworth, 1953), and it frequently causes death of the infected animals. For any drug to be considered of use for bovine trypanosomiasis in West Africa, it must possess high activity against both *T. vivax* and *T. congolense*.

Materials and Methods

"528."—The drug used was a sample of the chloride kindly supplied for trial by Dr. F. Hawking. The substance was dissolved in water at 80° C.; the orange-coloured solution was allowed to cool to room temperature and water added to adjust the concentration to 2%. When solutions of the drug were exposed to light, the colour darkened somewhat after two weeks, but no changes in toxicity or trypanocidal activity were detected in mice after one month. It was therefore concluded that solutions of the drug were reasonably stable.

Toxicity Test in Infected Cattle.—Seventeen Zebu cattle infected a few days previously with *T. congolense* (Nigerian strain A or B) were injected subcutaneously with doses of "528" ranging from 36 mg./kg. to 1 mg./kg. The dose was given on one side of the neck, or, if the volume was large, on both sides of the neck or into the brisket. The animals were observed continuously for 2–3 hr., and then on regular occasions until nightfall. Survivors were retained for 2–3 months and grazed out of doors during the day in order to observe any delayed toxic effects of the kind observed with phenanthridine derivatives (Goodwin and Chandler, 1951). The rectal temperature was taken before and at intervals after giving the drug. This test also served to give an indication of the curative activity of the drug.

Effect against T. congolense.—Fourteen Zebu cattle were inoculated subcutaneously with mouse blood containing *T. congolense* (Nigerian strain B). This organism had been maintained for one year in laboratory mice. Trypanosomes were detected in the blood of the cattle 12–15 days after inoculation. On the 18th day of the infection, doses of drugs were given subcutaneously. One group of animals was given antrycide methylsulphate as a standard, and a further group was kept untreated as controls. Fresh blood films were examined daily, and the site of inoculation was observed for evidence of local reaction.

Effect against T. vivax.—Twelve Zebu cattle were inoculated subcutaneously with the "Toro" strain of *T. vivax*. This strain was originally isolated from a horse and had been maintained by serial passage in sheep. Just before the experiment began the strain was transmitted from a sheep through *Glossina palpalis* to a cow. Blood from this cow was used to inoculate the experimental cattle.

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Trypanosomes appeared in the blood of all the animals 7 days after inoculation, and treatment was given on the 8th day. Injections of drugs were given subcutaneously to groups of three cattle. The cattle were kept in screened houses throughout the experiment, and blood films were examined daily for 6 weeks or longer. The site of injection of the drug was observed for local reaction.

A further experiment was made with a more recently isolated strain of *T. vivax*. This strain ("Bokkos") was passaged from the original bovine host by syringe into a sheep. Blood from the sheep was transferred to a cow, which was used as the donor of infected blood for the main experiment. (The donor was eventually treated with antrycide methylsulphate, and included in the results of the experiment.)

Eleven cattle were inoculated, and trypanosomes were detected in their blood 5 days later. Doses of drugs were given subcutaneously on the 9th day after infection. Six animals were given "528"; three, including the donor, were given antrycide methylsulphate; three were kept untreated as controls.

Development of Drug Resistance.—An attempt was made to produce a strain of *T. congolense* resistant to "528," using splenectomized mice. At each passage the infection was treated with a dose of drug sufficient to cause temporary disappearance of parasites from the blood. When the infection relapsed trypanosomes were transferred to clean, splenectomized mice. A total of 18 passages were made in this way, and the sensitivity of the trypanosome to the drug re-determined.

RESULTS

Toxicity

The local reaction at the site of injection of "528" was always comparatively mild and was similar to that caused by antrycide methylsulphate. A swelling, which was not much larger than the volume of drug given, remained for 3 or 4 days and then resolved slowly. The chief toxic effects observed immediately after injection were profuse salivation, muscular tremors, and incoordination. Animals often lay down and were unable to rise unassisted for 10 min. or more.

All animals given 8 mg./kg. or more died, the survival time varying from 12 hr. to 9 weeks. Of the five cattle which received 5 mg./kg. of "528," two died in 4–8 weeks. Doses of 3, 2, and 1 mg./kg. did not cause death; but the immediate toxic effects of 3 mg./kg. were serious enough to make the use of such doses undesirable in the field.

Post-mortem examination of the cattle which died showed the main lesion to be an acute nephritis similar to that produced by toxic doses of antrycide methylsulphate. Red streaks, due to masses of erythrocytes in the tubules, were visible macroscopically in both cortex and medulla. The glomeruli were packed with erythrocytes; the cells of the convoluted tubules showed karyolysis and many had ruptured into the lumen; a hyaline substance was present in many tubules. The collecting tubules of the medulla showed similar but less marked changes.

Curative Effect against T. congolense

During the toxicity trials in infected cattle, it was observed that *T. congolense* disappeared from the blood of cattle injected with 1 mg./kg. of "528." The doses used in the subsequent experiment gave the following results:

1 mg./kg. or 0.5 mg./kg. of "528": Apparently cured the three cattle treated at each dose level.

0.25 mg./kg.: One animal was apparently cured; one showed a scanty infection (on one day only) four weeks after treatment; the third relapsed 14 days after treatment and died of trypanosomiasis seven weeks later.

0.5 mg./kg. of antrycide methylsulphate cured one of the two animals treated; the other relapsed four weeks after treatment. Untreated control animals showed regular but not heavy infections; one died after 8 weeks, one after 5 months, and the third was killed to provide histopathological specimens. It is clear that "528" is effective against this strain of syringe-transmitted *T. congolense* and has a wide margin of safety.

Effect against T. vivax

With the "Toro" strain, two of three cattle relapsed, 10 and 16 days respectively, after a dose of 2 mg./kg. of "528." The third animal was apparently cured.

After a dose of 1 mg./kg. all three cattle relapsed within nine days of treatment, and all showed large numbers of parasites in the blood.

After treatment with "528," trypanosomes remained in the blood on the day following the dose, and then disappeared until relapse occurred.

Three cattle treated with 5 mg./kg. of antrycide methylsulphate were apparently cured.

Three controls showed heavy blood infections during the first four weeks, after which the disease became chronic in type.

With the "Bokkus" strain, all of six cattle injected with 2.5 mg./kg. of "528" relapsed within 11 days of treatment, and one died. Three animals which were given 5 mg./kg. of antrycide methylsulphate were apparently cured and no trypanosomes were observed again in the peripheral blood; one of these cattle died 18 days after drug treatment, and showed lesions of helminthiasis and unresolved trypanosomiasis.

Two of three control animals died of the acute trypanosome infection; the third was killed during the acute infection to obtain specimens. The "Bokkus" strain of *T. vivax* was virulent, and gave very heavy blood infections.

These experiments show clearly that "528" has no appreciable curative effect against *T. vivax*, even when used in doses approaching the toxic level.

Drug Resistance

Doses up to 1 mg./kg. of "528" given to normal mice infected with *T. congolense* were insufficient to prevent relapse. After 18 treated passages in splenectomized mice, relapses occurred after a dose of 4 mg./kg. However, after one passage in normal mice, resistance was hardly detectable. No information is available upon the readiness with which the drug produces resistance in infected cattle.

Discussion

The results of this investigation show clearly that, although "528" had an appreciable action against a syringe-transmitted strain of *T. congolense*, it had no curative effect against two strains of *T. vivax*. "528" would therefore be of very limited value in the treatment of cattle trypanosomiasis in West Africa, where *T. vivax* is the more important cause of cattle disease.

The drug is of interest because, like the phenanthridinium derivatives and antrycide, it is a quaternary heterocyclic compound, and it has toxic effects which are similar to those of antrycide.

I wish to thank Colonel H. W. Mulligan, formerly Director, West African Institute for Trypanosomiasis Research, and Dr. K. Unsworth for the help and advice which they gave in connexion with this investigation. The work was submitted as part of a thesis for the degree of Ph.D. in the University of London.

References

Goodwin, L. G., and Chandler, R. L. (1952). *Brit. J. Pharmacol.*, **7**, 591.

Keneford, J. R., Lourie, E. M., Morley, J. S., Simpson, J. C. E., Williamson, J., and Wright, P. H. (1948). *Nature, Lond.*, **161**, 603.

Lourie, E. M., Morley, J. S., Simpson, J. C. E., and Walker, J. M. (1951). *Brit. J. Pharmacol.*, **6**, 643.

Unsworth, K. (1953). *Ann. trop. Med. Parasit.*, **47**, 361.

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PHARMACOLOGICAL ACTIONS OF PURE MUSCARINE CHLORIDE

BY

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The action of chromatographically pure crystalline muscarine chloride, prepared from *Amanita muscaria*, has been compared with acetylcholine chloride (ACh) on a number of different organs from a variety of species. Muscarine caused spasm *in vivo* and *in vitro* of muscles of the gut, uterus, urinary bladder, and bronchus. It also caused contraction of the horse ureter and carotid artery chain *in vitro* and slowed the isolated auricles of the guinea-pig and rabbit, and the frog heart.

Muscarine caused a drop in blood pressure, although *in vitro* it produced either constriction or dilatation of the blood vessels of the rabbit ear.

All these actions resembled those of acetylcholine, though muscarine was usually more potent. Muscarine effects were readily prevented by atropine sulphate. It had a slight action on the frog rectus abdominis muscle, causing a contracture at high concentrations. Muscarine was destroyed neither by pepsin nor by boiling at any *p*H. It was inactive by mouth in a monkey in a quantity many times that which would cause poisoning by ingestion of *Amanita muscaria* in the human being. Muscarine neither inhibited nor was hydrolysed by either true- or pseudo-cholinesterase. Muscarine chloride did not cause paralysis of the neuromuscular junctions of the rat diaphragm or of the cat gastrocnemius.

Since Schmiedeberg and Koppe's paper in 1869, muscarine has been studied extensively. In the early literature, the term muscarine was applied not only to the active principle obtained from *Amanita muscaria*, but also to a compound derived from the action of nitric acid on choline which had a similar action. At one time these compounds were thought to be identical and the resulting confusion is well shown in Fühner's review on muscarine (1923).

A small quantity of crystalline, highly purified muscarine chloride was recently prepared from *Amanita muscaria* by Dr. S. Wilkinson. Its pharmacological actions have been compared with those of acetylcholine and are reported in the present paper.

METHODS

Experiments in vitro

All isolated tissues were bathed in appropriate oxygenated physiological saline solutions at 37° C., unless otherwise indicated.

Intestine.—Segments of ileum 3 to 5 cm. long were taken from the mouse, guinea-pig, and rabbit. Longitudinal strips (3 to 5 cm. by 3 to 5 mm.) from the dog, horse, and monkey were used. A circular strip from the horse was also tested. Similar lengths of rat colon and frog duodenum were also used. Frog intestine was examined in frog Ringer at room temperature.

Uterus.—Longitudinal strips from the mouse, guinea-pig, rabbit, rat, horse, and dog, and a circular strip from the horse were used. Mouse uteri were tested at 34° C.

Bladder.—Longitudinal strips of bladder from the guinea-pig, dog, rat, and monkey, and longitudinal and circular strips from the rabbit and horse were used. Strips from the frog bladder were tested at room temperature.

Tracheal Chain.—Chains, prepared from circular sections of the trachea of the guinea-pig and rabbit, were suspended in oxygenated van Dyke and Hastings solution at 37° C.

Arterial Chain.—These were made from circular sections of the horse carotid artery at 37° C.

Ureter.—Strips of ureter (4 to 5 cm.) from the horse were used.

Auricle.—The beating auricle from the guinea-pig or the rabbit was suspended in an oxygenated physiological saline at 30° C.

Heart.—The Clark (1912) frog heart preparation was set up at room temperature and perfused with frog Ringer.

Frog Rectus.—This was suspended in oxygenated frog Ringer at 5 to 10° C.

Leech.—The dorsal muscle of the leech was suspended in oxygenated Ringer at room temperature.

Ear.—The isolated ear of the rabbit was perfused at constant pressure with 0.9% NaCl solution at room temperature and the rate of venous outflow recorded.

Diaphragm.—The isolated rat phrenic nerve diaphragm preparation of Bülbring (1946) was immersed in oxygenated physiological saline solution at 37° C., and the nerve was stimulated by single rectangular pulses of 2 V. with a duration of 0.23 msec. at the rate of 5/min.

Experiments in vivo

Respiration.—Respiration was recorded from a cannula in the trachea of the rabbit or cat.

Blood Pressure.—The blood pressure was recorded manometrically from a cannula in the carotid artery of the rabbit, cat, or dog.

Uterus.—Movements of the non-pregnant uterus of the rabbit, cat, or dog were recorded by connecting the uterus *in situ* by a thread to a weighted lever, the peritoneal cavity being filled with saline at 37° C.

Bladder.—Changes in intravesical pressure in the cat or dog were recorded by tying a catheter through the bladder neck and connecting it to a wide bore saline reservoir and a tambour recorder.

Intestine.—Contractions of the longitudinal muscles of the intestines of the cat or dog were recorded by anchoring one end of a length of intestine to the abdominal wall and attaching the free end by a thread to a weighted lever. Contractions of the circular muscle were recorded by inserting a water-inflated balloon into a section of intestine and connecting it to a tambour recorder.

Gastrocnemius.—The sciatic nerve of the cat was stimulated maximally by single stimuli from a neon stimulator at 6 discharges/min.; contractions of the gastrocnemius were recorded by a thread from the muscle to a spring-loaded lever. Injections were made into the femoral vein.

Bronchoconstriction.—This was recorded in the guinea-pig or rabbit. Spontaneous respiration was abolished by an overdose of pentobarbitone sodium and the animal maintained on artificial respiration. Bronchoconstriction was recorded as a decrease in intrathoracic pressure.

In all *in vitro* and *in vivo* experiments the activities of muscarine chloride and acetylcholine chloride were compared by producing, whenever possible, equivalent responses.

Anaesthetics.—For the bronchoconstriction test in rabbits or guinea-pigs, ether and pentobarbitone sodium were used. Otherwise, urethane was used for rabbits, and pentobarbitone sodium for cats and dogs.

Conscious Animal.—A monkey (female 6.7 kg.) was given muscarine by stomach tube and by intraperitoneal injection.

Toxicity.—Groups of 5 albino mice were used, injections being made into a tail vein.

Manometric Methods.—A Warburg respirometer with single side arm flasks was used. The substrate—acetyl-β-methylcholine chloride for true cholinesterase and benzoylcholine chloride for pseudo-cholinesterase—was placed in the side arm. The enzyme preparation—human red blood cells for true cholinesterase and human plasma for pseudo-cholinesterase—was placed in the base of the flask with muscarine chloride, and Ringer-bicarbonate solution was added, bringing the total volume to 0.3 ml. Final concentrations were: acetyl-β-methylcholine, 0.03 M; benzoylcholine, 0.015 M; and muscarine chloride, 5.0×10^{-4} M. The whole system was gassed for 10 min. with a 95% N_2 and 5% CO_2 mixture. The muscarine and the enzyme were left in contact at 37° C. for 30 min. before tipping in the substrate. The rate of evolution of CO_2 was then compared with that of a control in the absence of muscarine.

Stability.—Muscarine chloride at concentrations of 1.0, 10.0, and 100.0 μg./ml. in 0.9% NaCl solution was stored in air at 0° C. for 18 months and its activity on the rabbit ileum was compared with a freshly prepared sample. Muscarine was boiled at *p*H 1.0, 6.0, and 11.0 for 10 min. and similarly tested. It was also tested after digestion in pepsin (10.0 mg./ml.) at *p*H 3.0 at 37° C. for 18 hr., and also after incubation in human oxalated blood at 37° C. for 1 hr.

Results

Stability.—Muscarine chloride showed no loss of potency when stored in solution for 18 months at 0° C. It was stable to boiling for 10 min. at *p*H 1.0, 6.0, and 11.0; resistant to peptic digestion at *p*H 3.0 at 37° C. for 18 hr., and showed no loss of activity when incubated in human oxalated blood at 37° C. for 1 hr.

Toxicity.—The mouse i.v. LD50 for muscarine chloride was calculated to be 0.23 mg./kg. and for acetylcholine chloride 33.05 mg./kg., thus muscarine was 143 times more toxic than acetylcholine. Signs of poisoning were similar, acetylcholine being lethal almost immediately and muscarine after 3 to 10 min. Death never occurred later than 10 min. after the injection.

Experiments on Isolated Organs

Intestines.—Both muscarine and acetylcholine caused spasm of the isolated gut from the eight species tested. Sensitivity varied considerably between species (Table I) and between specimens from the same species. Atropine sulphate always blocked the responses to both compounds. Muscarine spasm usually developed more slowly than that caused by acetylcholine and took longer to recover after washing the preparation. Except on the mouse ileum and the rat colon, muscarine was

TABLE I

COMPARISON OF SPASMOGENIC ACTIVITIES OF MUSCARINE AND ACETYLCHOLINE ON ISOLATED INTESTINAL MUSCLES OF VARIOUS SPECIES

Species	Preparation	Equiactive Concentrations (mμg./ml.)		Activity Ratio of Muscarine to ACh (ACh=1)	
		ACh	Muscarine		Mean
Mouse ..	Ileum	33·0	40·0	0·83	0·83
		330·0	400·0	0·83	
Guinea-pig	,,	7·5	3·0	2·5	
		10·0	4·0	2·5	4·1
		15·0	10·0	1·5	
		200·0	20·0	10·0	
Rabbit ..	,,	10·0	10·0	1·0	
		40·0	30·0	1·3	2·6
		100·0	33·0	3·0	
		100·0	20·0	5·0	
Dog ..	,,	33·0	8·0	4·1	
	(longitudinal)	33·0	8·0	4·1	4·1
Rat ..	Colon	166·0	155·0	1·0	
		33·0	66·0	0·5	0·75
Frog ..	Duodenum	3,300·0	100·0	33·0	
		1,650·0	50·0	33·0	33·0
Horse ..	Ileum	16·6	12·5	1·3	
	(longitudinal)	330·0	10·0	33·0	
		330·0	10·0	33·0	22·5
,, ..	Ileum	330·0	6·0	55·0	
	(circular)	1,000·0	6·0	167·0	111·0
Monkey ..	Ileum	100·0	10·0	10·0	
	(longitudinal)	60·0	6·0	10·0	10·0

TABLE II

COMPARISON OF SPASMOGENIC ACTIVITIES OF MUSCARINE AND ACETYLCHOLINE ON ISOLATED UTERINE MUSCLES OF VARIOUS SPECIES

Species	Preparation	Equiactive Concentrations (mμg./ml.)		Activity Ratio of Muscarine to ACh (ACh=1)	
		ACh	Muscarine		Mean
Mouse ..	Longitudinal	1,000·0	1,000·0	1·0	1·0
		166·0	150·0	1·1	
		1,660·0	1,660·0	1·0	
Guinea-pig	,,	666·0	100·0	6·7	
		333·0	83·0	4·0	5·3
Rabbit* ..	,,	250·0	666·0	0·37	
		166·0	333·0	0·5	0·43
Rat ..	,,	500·0	300·0	1·67	
		267·0	234·0	1·14	
		333·0	84·0	4·0	2·27
Horse ..	,,	67·0	10·0	6·7	
		166·0	25·0	6·7	
		1,000·0	100·0	10·0	
		1,670·0	167·0	10·0	8·35
,, ..	Circular	100·0	12·0	8·5	
		500·0	50·0	10·0	
		1,000·0	100·0	10·0	
		670·0	67·0	10·0	
		330·0	33·0	10·0	9·7
Dog ..	Longitudinal	1,000·0	100·0	10·0	

* Muscarine abolished ACh response temporarily.

more active than acetylcholine in producing spasm; again, except on these two tissues, physostigmine increased the sensitivity of the muscle to acetylcholine, and the ratio of activities of the two compounds became unity. In the two exceptional preparations, physostigmine did not always cause an increase in the sensitivity to acetylcholine.

Uterus.—Table II gives the results on the isolated uterus from six different species. As on the gut, the spasm caused by muscarine was slower in onset and recovery after washing was slower than with acetylcholine. Atropine prevented the action of each compound. Muscarine was more active than acetylcholine except on the uterus of the mouse and of the rabbit. An interesting finding with the rabbit uterus preparation was that, after recovery from a muscarine-induced spasm, the response to acetylcholine was temporarily abolished.

Bladder.—In all seven species tested muscarine was more active than acetylcholine (Table III). Muscarine responses were slower and were prevented by atropine.

TABLE III

COMPARISON OF SPASMOGENIC ACTIVITIES OF MUSCARINE AND ACETYLCHOLINE ON ISOLATED MUSCLES FROM THE URINARY BLADDER OF VARIOUS SPECIES

Species	Preparation	Equiactive Concentrations (mμg./ml.)		Activity Ratio of Muscarine to ACh (ACh=1)	
		ACh	Muscarine		Mean
Guinea-pig	Longitudinal	133·0	12·0	8·3	
		1,000·0	58·0	17·2	
		667·0	18·0	37·0	20·8
Rabbit ..	,,	130·0	4·0	32·5	
		160·0	8·0	20·0	
		333·0	12·0	27·8	
		100·0	6·0	16·6	
		333·0	12·0	27·8	24·9
,, ..	Circular	667·0	13·0	51·3	
Dog ..	Longitudinal	333·0	13·0	25·3	
		167·0	3·0	55·8	
		167·0	2·0	83·5	
		333·0	3·0	111·0	
		333·0	2·0	167·0	88·5
Rat ..	,,	67·0	17·0	3·9	
		200·0	23·0	8·7	6·3
Frog ..	,,	12·0	5·7	2·0	
		6·7	3·3	2·0	
		6·7	2·8	2·4	
		200·0	33·0	6·0	
		167·0	16·7	10·0	4·5
Horse ..	,,	33·0	3·3	10·0	
		66·0	6·6	10·0	10·0
,, ..	Circular	50·0	6·7	7·5	
		33·0	3·3	10·0	8·8
Monkey ..	Longitudinal	1,330·0	6·7	200·0	
		1,000·0	5·0	200·0	200·0

Other Isolated Organs.—Table IV summarizes the results obtained with other preparations.

With the tracheal chain preparation of the guinea-pig and of the rabbit, muscarine was many times more active than acetylcholine in causing spasm. Its action was slower with a latent period of about 2 to 3 min., an effect not seen with acetylcholine.

The difference in the rate of action was even more marked in the contraction of the carotid artery chain of the horse in which the spasmogenic action was not apparent until 5 min. after muscarine was added to the bath.

TABLE IV

COMPARISON OF SPASMOGENIC ACTIONS OF MUSCARINE AND ACETYLCHOLINE ON OTHER ISOLATED ORGANS FROM VARIOUS SPECIES

Species	Preparation	Equiactive Concentrations (mμg./ml.)		Activity Ratio of Muscarine to ACh (ACh=1)	
		ACh	Muscarine		Mean
Guinea-pig	Tracheal chain	3,000·0	50·0	60·0	
		1,670·0	6·7	250·0	155·0
Rabbit ..	,,	1,670·0	6·7	250·0	
Horse ..	Carotid artery chain	1,000·0	400·0	2·5	
		500·0	150·0	3·3	2·9
,, ..	Ureter	1,000·0	216·0	4·6	
		1,000·0	13·0	77·0	
		1,000·0	234·0	4·3	28·6
Guinea-pig	Auricle	16·7	16·7	1·0	
		33·0	40·0	0·83	
		25·0	16·7	1·5	1·1
Rabbit ..	,,	200·0	30·0	6·7	
		400·0	40·0	10·0	
		100·0	27·5	3·6	
		100·0	50·0	2·0	
		200·0	35·0	5·7	5·6
Frog ..	Heart	13·4	20·0	0·67	
,, ..	Rectus	50·0	10,000·0	0·005	

The ureter of the horse contracted well to both compounds and recovery of the preparation after washing out either compound was rapid.

Muscarine and acetylcholine were equiactive in producing slowing of the rate and decrease of the size of the beat of the isolated guinea-pig auricle ; on the rabbit auricle, muscarine was 5.5 times more active than acetylcholine. Fibrillation of the rabbit auricle was converted by both drugs to a slow regular beating of the organ. Muscarine was 10 times more potent than acetylcholine and, after washing, the period before fibrillation recurred was 14 times longer.

On the isolated frog heart, both muscarine and acetylcholine caused a slowing of the rate and a decrease in the amplitude of the beat in both the auricle and the ventricle. Recovery was slow and never quite complete.

All the above effects were prevented by atropine. The action of atropine on the fibrillating auricle was not tested.

An unexpected finding was that muscarine in a concentration of 10,000 mμg./ml. caused a very slow contracture of the isolated frog rectus abdominis muscle equivalent in height to the much more rapid response caused by 50 mμg./ml. acetylcholine. The muscle was slow to recover after washing out the muscarine. The responses to both drugs were prevented by (+)-tubocurarine.

On the dorsal muscle of the leech, a response to acetylcholine could only be obtained after physostigmine. No response was obtained with muscarine in concentrations up to 1,000 mμg./ml. alone or after physostigmine.

In the isolated perfused rabbit ear, muscarine and acetylcholine were equiactive, both producing constriction or both producing dilatation of the blood vessels in any one ear.

Muscarine in concentrations up to 10,000 mμg./ml. had no action on the rat phrenic nerve diaphragm preparation *in vitro,* nor did it influence the paralysis caused by (+)-tubocurarine or suxamethonium.

Experiments in vivo

Respiration.—Both drugs caused temporary decrease of the respiratory movements in the rabbit or cat. Muscarine was four times more active than acetylcholine in both species.

Blood Pressure.—Again muscarine was about four times more potent than acetylcholine in causing a fall in blood pressure in the rabbit, cat, or dog. The response to muscarine was slower and more prolonged than that with acetylcholine.

Organs.—Muscarine caused contractions of the non-pregnant uterus of the rabbit, the cat, or the dog, the urinary bladder of the cat or dog, and both circular and longitudinal muscles of the gut of the cat or dog. Acetylcholine acted similarly to but was less active than muscarine. The organs of the dog were particularly sensitive to muscarine. This accounts for the much greater activity ratio of muscarine to acetylcholine in this species than in the other species studied (Table V).

TABLE V

COMPARISON OF VARIOUS ACTIONS OF MUSCARINE AND ACETYLCHOLINE *IN VIVO*

Action on	Species	Type of Response	Mean Activity Ratio of Muscarine to ACh (ACh = 1)	Minimum Active Dose of Muscarine i.v. (μg./kg.)
Intrathoracic pressure	Guinea-pig	Bronchoconstriction	5·0	2·0
	Rabbit	,,	4·5	2·0
Respiration ..	Rabbit	Depression	4·0	5·0
	Cat	,,	4·0	5·0
Blood pressure	Rabbit	Fall	4·0	5·0
	Cat	,,	5·0	1·0
	Dog	,,	Approx. 3·5	0·5
Uterus ..	Rabbit	Contraction	4·0	10·0
	Cat	,,	>4·0*	5·0
	Dog	,,	Approx. 250·0	0·5
Bladder ..	Cat	,,	20·0	0·5
	Dog	,,	Approx. 300·0	0·1
Gut (circ.) ..	Cat	,,	>4·0*	5·0
	Dog	,,	Approx. 40·0	0·5
Gut (long.) ..	Cat	,,	>10·0*	2·0
	Dog	,,	Approx. 500·0	0·2

* No response obtained with acetylcholine.

No response from the uterus and gut of the cat was obtained with acetylcholine up to 20 μg./kg. The cat bladder proved interesting in that the response to acetylcholine was greatly reduced after a dose of muscarine.

Atropine sulphate prevented all responses obtained with both drugs *in vivo*.

The cat gastrocnemius was not paralysed by muscarine or by acetylcholine.

In the guinea-pig and rabbit, bronchoconstriction, obtained with both muscarine and acetylcholine, could be prevented by atropine. Recovery from both drugs was spontaneous, but that from muscarine was slower than with acetylcholine.

The following experiment on a monkey was of interest as it showed that muscarine apparently had little activity by mouth. An intraperitoneal injection of 2 mg. of muscarine was rapidly followed by profuse mucous salivation and vomiting. The animal became prostrate with apparently severe abdominal discomfort. There was miosis, and respiration was deep and slow with uncoordinated abdominal and thoracic movements. Atropine completely abolished all these signs within 5 min. with the exception of the miosis, which gave place to mydriasis after about 40 min.

A fortnight later, this monkey was given 2 mg. muscarine by mouth and no signs of poisoning developed during the next 5 hr. Then, 0.5 mg. was given intraperitoneally and severe toxic effects occurred which were abolished by atropine.

Action on Cholinesterases.—At high concentrations, acetylcholine inhibits both true and pseudocholinesterase (Alles and Hawes, 1940). Muscarine at a concentration of 5.0×10^{-4} M had no inhibitory action on either true or pseudocholinesterase, nor was it hydrolysed by either enzyme.

DISCUSSION

The actions of pure muscarine chloride have been shown to conform to the type of actions of acetylcholine and other drugs known as "muscarinic," that is, "acting on smooth muscles and glandular cells that are innervated by postganglionic cholinergic nerve fibres." This study has been largely limited to a quantitative and qualitative comparison of the actions of muscarine chloride and acetylcholine chloride on smooth muscle systems.

Both *in vitro* and *in vivo*, muscarine caused spasm of the gut, uterus, and urinary bladder of a number of different species. Muscarine was not hydrolysed by cholinesterases. It was usually more active than acetylcholine w/w, and its action was always slower and more prolonged. It caused constriction of the bronchi of the guinea-pig and rabbit and spasm of the horse ureter and carotid artery *in vitro*. This last observation was of interest as muscarine is generally considered to be a vasodilator. However, in the isolated rabbit ear, muscarine caused either constriction or dilatation of the blood vessels, but, *in vivo* in the cat and dog, it caused a marked drop in blood pressure; it slowed the isolated frog heart and the isolated auricles of the guinea-pig and rabbit. All these actions were shared with acetylcholine and were readily prevented by atropine sulphate. In most preparations tested, treatment with physostigmine increased the sensitivity of the muscle to acetylcholine, and its activity then became comparable to that of muscarine although it remained faster in action. Physostigmine never altered the sensitivity of the isolated muscle to muscarine as has been claimed by Waser (1955) to occur *in vivo*.

At the neuromuscular junction it has not been possible to demonstrate any action of muscarine in experiments on the rat diaphragm, on the cat gastrocnemius preparation, or on the dorsal muscle of the leech, thus confirming the results of

Ambache, Perry and Robertson (1956) and of Zaimis (quoted by these authors). On the other hand the frog rectus abdominis differed from the other muscles in that 10,000 mμg./ml. of muscarine produced a slow contracture. Ambache, Perry, and Robertson (personal communication) did not employ such high concentrations of muscarine.

Although muscarine was stable to boiling and to pepsin, no action by mouth was found in the monkey despite the fact that the amount given was many times that which would cause poisoning by the ingestion of *Amanita muscaria* in the human being.

I am indebted to Dr. S. Wilkinson for the preparation of muscarine chloride from *Amanita muscaria* made available through the officers of the Forestry Commission. I wish to acknowledge the help and advice given by Dr. A. C. White, and the competent technical assistance of Miss L. S. Brooks, Miss S. M. Scammell, Mr. F. Huggins, and Mr. D. J. Tulett.

References

Alles, G. A., and Hawes, R. C. (1940). *J. biol. Chem.*, **133**, 375.

Ambache, N., Perry, W. L. M., and Robertson, P. A. (1956). *Brit. J. Pharmacol.*, **11**, 442.

Bülbring, E. (1946). Ibid., **1**, 48.

Clark, A. J. (1912). *Proc. Roy. Soc. Med.*, **5**, 181.

Führer, H. (1923). *Die Muscaringruppe*, Heffters Handbuch. Bd. 1, pp. 640–683. Berlin: Springer.

Schmiedeberg, O., and Koppe, R. (1869). *Das Muscarin das giftige Alkaloid des Flugenpilzes* (Agaricus muscarius L.). Leipzig: Vogel.

Waser, P. G. (1955). *Experientia*, **11**, 452.

Brit. J. Pharmacol. (1957), **12**, 53.

THE ASSAY OF ANTI-ACETYLCHOLINE AGENTS FOR ANTAGONISM OF PILOCARPINE-INDUCED SALIVATION IN RABBITS

BY

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Experimental conditions affecting tests of atropine-like agents for antagonism of pilocarpine-induced salivation in rabbits have been examined. A simple method of assay of such agents is described. It gave results of fair accuracy and reproducibility, permitted a full statistical analysis, and provided an estimate of the error.

The effect of drugs on salivary flow has been examined in experimental animals by several techniques. Cushny (1920) used dogs with salivary fistulae, and Bülbring and Dawes (1945) measured the flow of saliva from the submaxillary gland of anaesthetized cats by cannulation of Wharton's duct. Issekutz (1917), who collected the saliva which dripped from the mouths of rabbits under urethane, reported that 1.5 to 2.0 mg. atropine stopped pilocarpine-induced salivation. This last method, which requires little technical skill, has been adapted by many workers, but there has been little detailed analysis of the optimal conditions. Furthermore, statistical evaluation of the results has rarely been attempted and the accuracy of the methods is therefore virtually unknown. Brown and Werner (1949), indeed, found such variability between responses of different rabbits that they regarded any quantitative assay as impracticable.

This paper describes conditions that affect the responses and the accuracy of the assay and outlines a simple method which gives results whose accuracy can be readily and fully assessed.

Analysis of Factors Influencing Salivary Flow

Use of Urethane.—Unlike several workers who used unanaesthetized animals (e.g., Fromherz, 1933; Lands, Nash, and Hooper, 1946; Chen, 1954), we could not regularly obtain a suitable amount of saliva from rabbits which had received no urethane. However, a large oral dose of urethane 1 g./kg. or more was undesirable, for the narcotic action lasted some 6 to 10 hr., and the animals lost their cough and swallowing reflexes completely (Brown and Werner, 1949; Brown, Thompson, Klahm, and Werner, 1950; Hoekstra, Tisch, Rakieten, and Dickison, 1954; Tripod, 1949). The dose of urethane is therefore critical.

The variation in the amount of saliva collected in 30 min. with the dose of urethane was determined after various doses of pilocarpine, for one set of eight rabbits (Fig. 1). The drugs were given in random order, over 4½ weeks, each animal receiving every possible combination. At all three doses of pilocarpine, the slopes of the urethane dose-response lines were very steep. Indeed, the urethane dose appeared to control the response more effectively than did the pilocarpine. The urethane probably did not affect the actual rate of salivation, but only increased the efficiency of collection.

An oral dose of 0.5 to 0.625 g./kg. of urethane was effectively sedative to the rabbits without producing loss of consciousness, and a good collection of saliva was possible. Collection was more efficient from rabbits given 1.0 g./kg. urethane, but, owing to the risk of harmful effects to the animals, 0.5 to 0.625 g./kg. was preferred.

The urethane was administered orally in 25 ml. saline to ensure adequate hydration and a good reserve of body water upon which to draw during and after the profuse salivation.

No other anaesthetic was tried in these investigations.

Dose of Pilocarpine.—Doses of pilocarpine used by previous workers in this field have ranged from 0.25 mg./kg. (Fromherz, 1933) to 100 mg./kg. subcutaneously (Graham and Lazarus, 1940). The relation between the dose of pilocarpine nitrate and the salivary response was investigated over the range of 1.25 to 5.0 mg./kg., at three

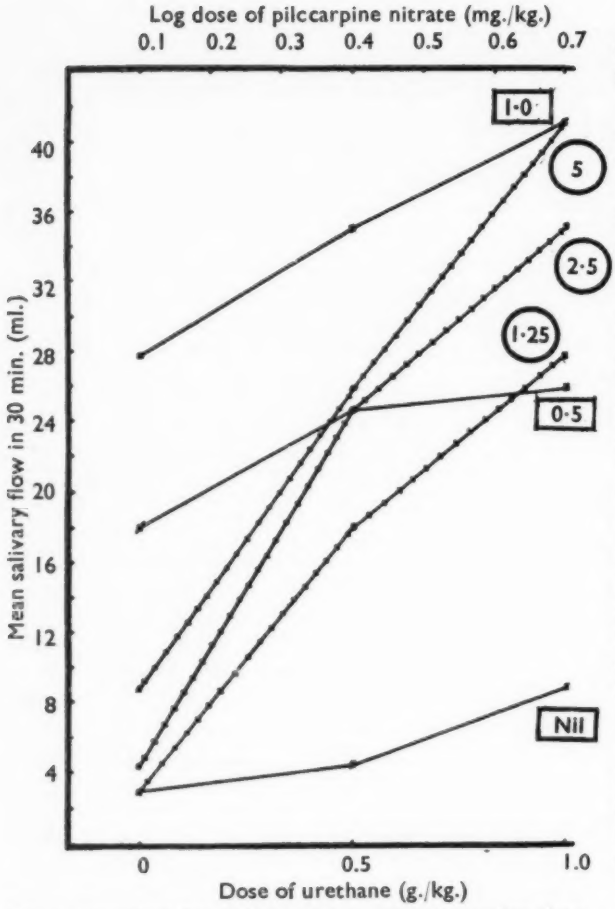

FIG. 1.—Combined dose-salivary response curves for various doses of urethane and pilocarpine, in a set of 8 rabbits. ———— Pilocarpine (log dose on top abscissa) after various doses of urethane (g./kg., in squares). ı—ı—ı—ı—ı Urethane (bottom abscissa) preceding various doses of pilocarpine (mg./kg., in circles).

different doses of urethane (Fig. 1). The pilocarpine nitrate was injected subcutaneously 30 to 45 min. after the oral dose of urethane. The rabbits were then placed in their stocks and the saliva which dripped from their mouths was collected.

In the absence of urethane, an increase in the dose of pilocarpine from 1.25 to 5.0 mg./kg. had little effect on the small amount of saliva obtained. On the other hand, with 0.5 g./kg. and 1 g./kg. of urethane, the response to even 1.25 mg./kg. pilocarpine was considerably more than that to 5 mg./kg. after no urethane. When 0.5 g./kg. of urethane was given, there was little difference between the amounts of saliva secreted after the injection of 2.5 mg. and 5 mg./kg. pilocarpine. However, the higher dose was used throughout the investigations, since, after it, salivation was of convenient duration (90 min.) and of suitable intensity—not too readily antagonized by drugs, yet susceptible enough to give a sensitive assay.

Choice of Animals.—Rabbits of 1.5 kg. and over were used. Differences in sex and weight did not appear to influence the response to the drugs.

The rabbits showed enormous variability in their sensitivity to atropine. In about half of them the salivary response to pilocarpine was not affected significantly by doses of atropine up to 80 μg./kg., given subcutaneously 15 min. before the pilocarpine. On the other hand the remainder showed about a 50% decrease in flow after 5 to 10 μg./kg., and almost a 100% decrease after 25 μg./kg. This insensitivity to atropine was presumably due to the presence in the blood of an enzyme—atropinesterase (Glick, 1940)—capable of hydrolysing atropine. Serum from several resistant rabbits was able to destroy atropine *in vitro*.

Although both Glick and Glaubach (1941) and Ammon and Savelsberg (1949) have reported widely ranging figures for the effective atropinesterase activity of the blood of those rabbits possessing it, we found that rabbits showed either a large (>50%) reduction in salivary flow after 25 μg./kg. atropine, or else an insignificant reduction (<20%) after double or treble that dose. Consequently, once a consistent salivary response to the pilocarpine alone had been obtained in a rabbit, a single test with 25 μg./kg. atropine given 15 min. before the pilocarpine was sufficient for the assessment of atropine resistance. All the animals showing less than a 50% inhibition of salivation by this dose were rejected.

Interval Between Administration of Atropine and of Pilocarpine.—In order to find the optimal time interval between the injection of atropine, or similar drugs, and of pilocarpine, a random design assay was performed on one set of eight rabbits, in which the antagonism of salivary flow was measured for four different atropine-like drugs given 0, 5, 15, 35, and, on two occasions, 70 min. before the injection of pilocarpine. The drugs used were atropine sulphate, atropine methyl nitrate, benactyzine (β-diethylaminoethyl benzilate HCl) and oxyphenonium (phenylcyclohexyl glycollic acid ester of β-diethylaminoethanol methobromide). The animals received 0.5 to 0.625 g./kg. urethane orally and 5 mg./kg. pilocarpine nitrate subcutaneously.

The mean percentage inhibitions of salivation are given in Table I and the time-response curves are plotted in Fig. 2.

Atropine appeared to exhibit maximal activity when administered about 15 min. before the pilocarpine; its effect after 35 min. was less than after 5 or 15 min. Atropine methyl nitrate had a more prolonged action which reached a maximum when it was given about 35 min. before pilo-

TABLE I

THE EFFECT OF VARYING THE INTERVAL BETWEEN THE INJECTIONS OF AN ATROPINE-LIKE DRUG AND OF PILOCARPINE ON THE INHIBITION OF SALIVARY FLOW

Drug	Dose μg./kg. s.c.	Mean % Inhibition of Salivation at Different Times (min.) Between Injection of Drug and Pilocarpine				
		0	5	15	35	70
Atropine SO_4 ..	5	32·6*	45·4	55·1	36·9*	
Atropine methyl nitrate	2	24·4*	35·6*	49·9	59·3	53·5
Benactyzine	750	36·5	30·5	24·5	22·3	
Oxyphenonium ..	5	48·9*	60·9	63·9	78·5*	78·5*

S.E. of each mean, 7·2.
* Response significantly different from that at 15 min. ($P<0.05$).

carpine. Even 70 min. after administration, atropine methyl nitrate still showed a greater effect than after 0 to 15 min. Oxyphenonium, the other quaternary compound, gave identical results. With benactyzine, however, maximal activity was obtained when the drug was given at the same time as pilocarpine.

It thus appears that the times at which different atropine-like drugs exert maximal activity after subcutaneous injection vary considerably. In the assay method described subsequently, the time between administration of the atropine-like drug and the pilocarpine was 15 min., in order to standardize conditions. The activity displayed under these conditions may not be maximal, particularly with compounds containing a quaternary nitrogen atom, and the relative potencies in terms of atropine might thus be smaller than could otherwise be obtained.

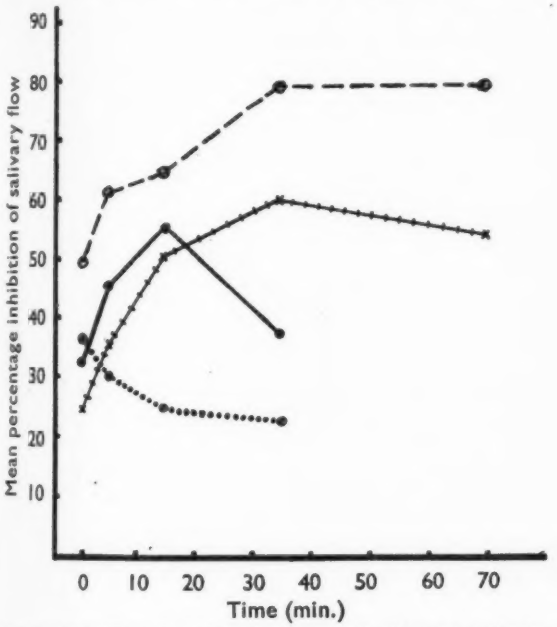

FIG. 2.—The effect of varying the time of injection of the atropine-like drug on the observed potency. Abscissa, time (min.) between injections of the drug and of pilocarpine (5 mg./kg.). Ordinate, mean percentage inhibition of salivary flow, for 8 rabbits. All drugs injected subcutaneously. ●————● Atropine sulphate 5 μg./kg. ×—ı—ı—ı—× Atropine methyl nitrate 2 μg./kg. ●·····● Benactyzine HCl 750 μg./kg. O — — — O Oxyphenonium bromide 5 μg./kg.

ANALYSIS OF RESULTS

Regular gradation of response with dose was shown in atropine-sensitive rabbits—that is, a regular decrease of salivary flow was obtained with increasing doses of atropine. The response of each animal was expressed as the percentage depression of the total volume of saliva collected after a dose of atropine or similar agent, compared with the mean of the volumes obtained from the same animal in two control experiments. The results obtained from each of eight animals in a set were averaged. In practice, control experiments were performed only once in every six to twelve experiments, the responses in all those tests falling between any two controls being expressed with reference to the mean of these two control values. The average standard deviation of such a mean control value, calculated from figures for each of eight rabbits tested over nine months, was 10.9 ml. Collection of saliva was maintained for 90 min. after the rabbits had received pilocarpine and had been placed in their stocks. By the end of this time, the flow had ceased in all except a few control observations where it had fallen to a rate of below 0.2 ml./min.

Eight animals for each experiment gave results of adequate accuracy. A set of animals could be used twice weekly, with only very occasional rests, for at least 15 months without any ill-effects or tolerance.

A large number of drugs could therefore be compared for their activity in antagonizing pilocarpine-induced salivation, on the same set of eight rabbits.

The time course of the salivation obtained from a set of eight rabbits in control experiments and in tests with various doses of atropine is given in Fig. 3. Where no atropine was given, the salivation response to pilocarpine appeared to fall away exponentially with time. If moderate to strong doses of atropine were given 15 min. before the pilocarpine, however, the time-log response lines appeared to curve. This might indicate that atropine was less readily eliminated or removed from the receptors than pilocarpine.

The dose-response relation for atropine was investigated several times, on two different sets of rabbits. Good approximations to a linear log dose-response line were obtained over the 20 to

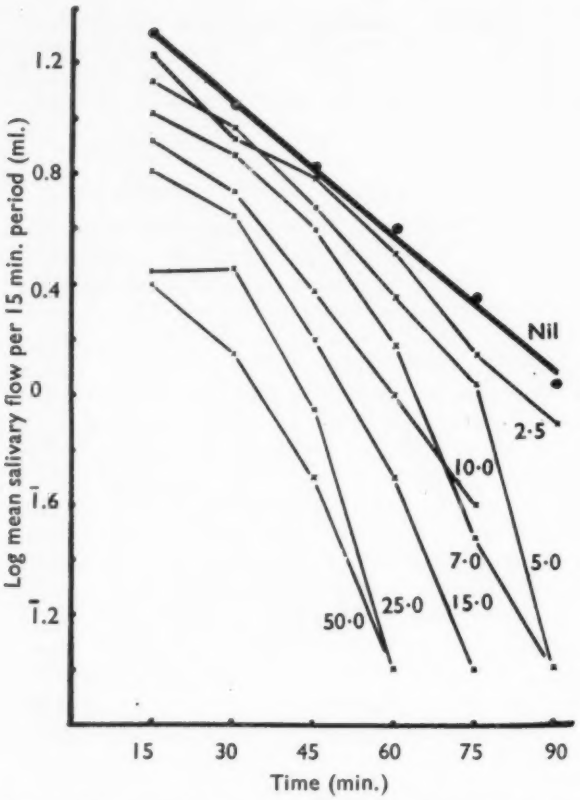

FIG. 3.—Time-salivary flow curves for a set of 8 rabbits, after 5 mg./kg. pilocarpine nitrate, preceded by various doses of atropine. Dose of atropine in μg./kg. is given alongside the appropriate line. Abscissa, time (min.) after injection of pilocarpine. Ordinate, mean log volume (ml.) of saliva for each rabbit, measured for 15 min. periods.

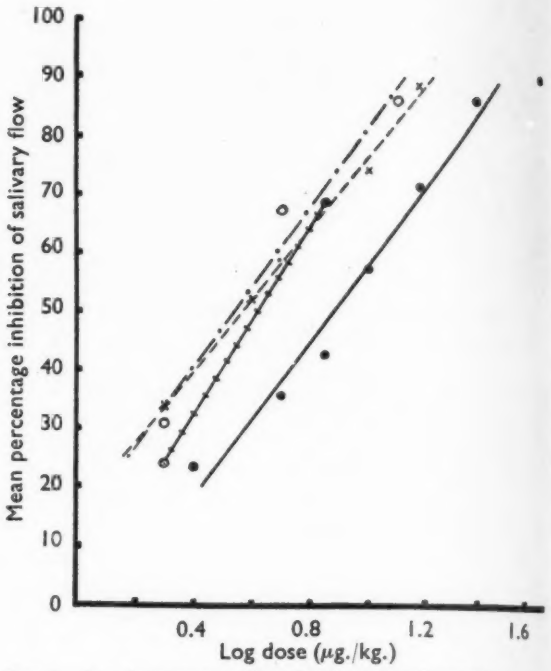

FIG. 4.—Log dose-response lines for 4 assays of atropine on two different sets of 8 rabbits. Regression lines calculated by method of least squares. ●———● Assay 5/55 on set A rabbits, × — — — × Assay 8/55, O — · — O Assay 2/56, ⊙—|—|—⊙ Assay 2/56 on set B rabbits.

90% maximal response range, and regression lines were fitted by the method of least squares (Fig. 4). The variance due to deviation from linearity was never statistically significant (i.e., $P<0.05$). Results were analysed by the standard analysis of variance method; one set of results is given in Table II. Although atropine sulphate was used, the doses were always calculated in terms of the base.

Table III gives data from five different assays, four on one set of rabbits and one on another. There is a close similarity of the results in Set B. The difference in the estimates of ED50 for atropine between the two sets of rabbits is largely an indication of differences encountered in the sensitivity of individual rabbits to atropine—even those apparently entirely lacking atropinesterase. The differences in response of these two sets of rabbits emphasize the need to compare anti-acetylcholine agents in the same group of rabbits. Many different compounds were so tested and

TABLE II

DOSE-INHIBITION OF SALIVATION DATA FOR ATROPINE ON ONE SET OF 8 RABBITS

Rabbit	% Decrease in Salivary Flow 15 min. after Atropine s.c.				Total
	Dose of Atropine (μg./kg.)				
	2	4	10	15	
1	−34	41	30	77	114
2	67	97	82	91	337
3	6	32	57	77	172
4	51	33	86	94	264
5	70	100	95	100	365
6	16	38	72	77	203
7	28	46	71	97	242
8	69	29	100	98	296
Total ..	273	416	593	711	1,993
Mean ..	34·1	52·0	74·1	88·9	

Slope $b=61{\cdot}1$. ED50 $=3{\cdot}6$ μg./kg.

ANALYSIS OF VARIANCE

Source of Variance	Sum of Squares	d.f.	Mean Square	F	P
Regression	13,903	1	13,903	39·2	<0·01
Deviation from linearity	65	2	32·5	10·9	>0·05
Between doses	13,968	3			
Between animals ..	12,488	7	1,784	5·0	0·01–0·1
Residual error	7,440	21	354·3		
Total	33,896	31			

$\lambda=0{\cdot}31$ where $\lambda=\frac{\sqrt{\text{Mean Square Error}}}{b}$ S.E. of $b=9{\cdot}7$.

TABLE III

RESULTS OF VARIOUS ASSAYS OF ATROPINE FOR INHIBITION OF SALIVATION

Rabbits:	Set A	Set B			
Assay No.	1	1	2	3*	4*
Date of Assay	5/55	8/55	2/56	2/56	4/56
No. of dose-levels used	6	4	3	2	2
Slope (b)	65·7	61·1	68·0	81·3	60·3
S.E.b	11·5	9·7	7·1	17·9	12·4
ED50 (μg./kg.)	7·5	3·6	3·4	4·2	5·1
Limits of error (P=0·05)	5·8–	2·9–	2·8–	3·5–	4·2–
of ED50	9·9	4·8	4·2	4·9	6·1
λ	0·40	0·31	0·20	0·30	0·22

*Part of a randomized design assay incorporating two other compounds.

their potency referred constantly to that of the standard (atropine), whose effective potency appeared to stay reasonably constant for at least nine months.

Results for Other Atropine-like Agents

In addition to atropine, several other anti-acetylcholine agents were tested by this method for their antagonism of induced salivation. Atropine methyl nitrate, oxyphenonium, and propantheline were tested on rabbits of Set B at about the same time as the second atropine assay (Table III); several doses were used in each assay. For all these compounds, doses were calculated in terms of the salt. The calculated regression lines are shown in Fig. 5, and the analysis of the results given in Table IV. The variance due to linear regression was highly significant ($P<0.01$) and that due to deviation from linear regression not significant ($P>0.2$).

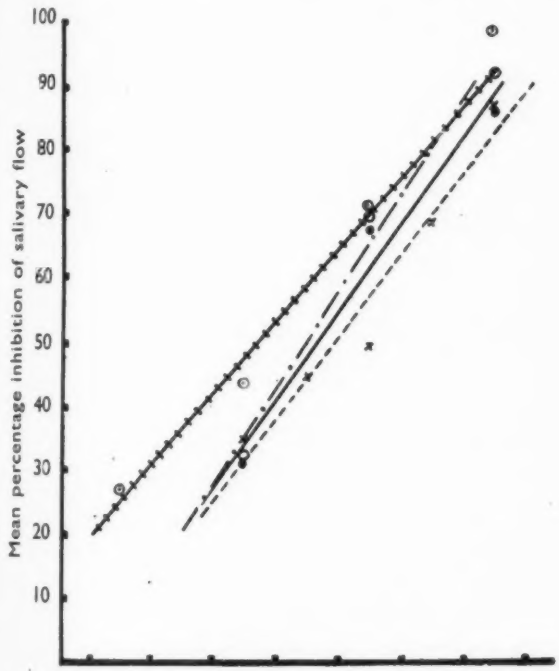

FIG. 5.—Log dose-response lines for: ●——● Atropine sulphate. ○—·—·—○ Atropine methyl nitrate. ×— · · —× Oxyphenonium. O — · — O Propantheline. All results obtained on same set of 8 rabbits.

TABLE IV

ASSAYS OF ATROPINE-LIKE DRUGS FOR INHIBITION OF SALIVATION

Compound	Atropine (Sulphate)	Atropine Methyl Nitrate	Oxyphenonium	Propantheline
No. of dose-levels tested	3	4†	5	3
Slope (b)	68·0	55·3	63·7	74·3
S.E.b	7·1	15·2	9·4	10·2
ED50 (μg./kg.)	3·4	2·2	3·9	3·2
Limits of error of ED50	2·8–	1·4–	3·2–	2·6–
(P=0·05)	4·2	3·6	4·8	4·0
Approx. relative potency*	1·0	1·5	0·9	1·1
λ	0·20	0·41	0·25	0·21

* $= \frac{\text{ED50 Atropine}}{\text{ED50 Compound}}$

† Only 3 used in the analysis, the fourth giving a response over 90% of maximal.

The relative potencies of the three compounds in terms of atropine are expressed as the ratios of their ED50s (Table IV). There was little difference in the activity of these four agents, although atropine methyl nitrate appeared slightly more potent than the tertiary base.

(2+2) Assay

The assessment of the relative potency of one drug in terms of another from dose-response data obtained at different times must, however, inevitably be liable to more error than that derived from tests carried out under identical conditions for both drugs. An assay method incorporating randomized doses was therefore adopted, in which, at any one time, the four pairs in a set of eight rabbits received different doses. Two doses of each compound giving respectively about a 35% and 70% response were administered

TABLE V

COMPARISON OF ACTIVITIES OF ATROPINE-LIKE DRUGS WITH THAT OF ATROPINE

	λ	Log Relative Potency (M)	S.E. of M	Rel. Potency	Limits of Error of Rel. Potency (P=0·95)
Assay 1:					
Combined b=65·6	0·30				
Atropine x atropine $MeNO_3$		0·357	0·104	2·28	1·40–3·70
x homatropine HBr		–2·039	0·109	0·0091	0·0055–0·0152
Assay 2:					
Combined b=59·8	0·22				
Atropine x tricyclamol Cl		–0·211	0·086	0·62	0·41–0·93

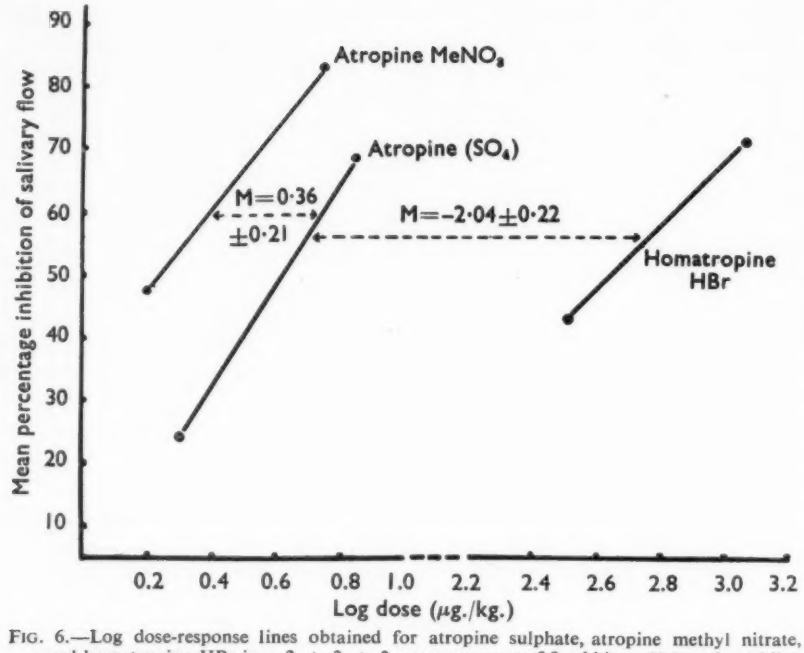

FIG. 6.—Log dose-response lines obtained for atropine sulphate, atropine methyl nitrate, and homatropine HBr in a 2 + 2 + 2 assay on a set of 8 rabbits. Values for M (log relative potency) ± its limits of error ($P=0.95$) are given, with atropine taken as standard.

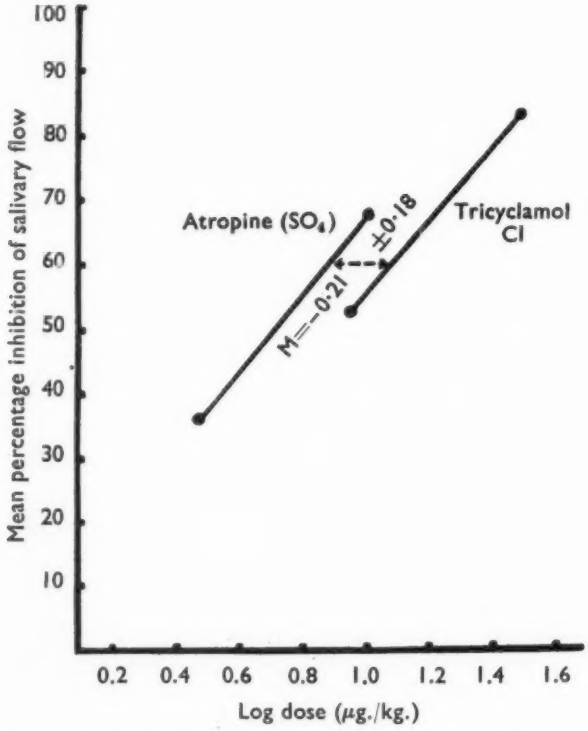

FIG. 7.—Log dose-response lines obtained for atropine sulphate and tricyclamol Cl in a 2 + 2 assay on a set of 8 rabbits. Log relative potency (M) ± its limits of error ($P=0.95$) are given, with atropine taken as standard.

subcutaneously. The ratio of the two doses was the same for all compounds in an assay. In the first assay, three compounds were tested—atropine sulphate, atropine methyl nitrate, and homatropine HBr. The doses were given in random order over six experiments. The assay results were compared with the mean result of two control experiments performed just before and just after the assay. In a second assay, tricyclamol chloride was tested against atropine in a similar manner. Analysis of results followed the same lines as before, with adaptations of the Schild (1942) and Holton (1948) methods of evaluating 2+2 assays.

The dose-response lines obtained in these assays can be seen in Figs. 6 and 7, and the relevant data tabulated in Table V. Variance due to regression was always highly significant ($P<0.01$), whereas that due to deviation from parallelism was not ($P>0.2$).

The relative potencies of atropine methyl nitrate, homatropine HBr, and tricyclamol in terms of atropine were 2.28, 0.0091, and 0.62 respectively; the difference in activity between the test compound and atropine was statistically significant with all the drugs.

DISCUSSION

Rabbits fully sensitive to atropine are essential for a reliable assay, since the dose of atropine producing 50% inhibition of salivation in about half the animals tested was more than ten times that necessary in the other half. Serum from the former rabbits destroyed atropine *in vitro*, whereas serum from the atropine-sensitive rabbits did not. Other atropine-like drugs are also susceptible to destruction in the serum of certain rabbits; homatropine, for example, is almost as readily attacked as atropine (Ammon and Savelsberg, 1949). On the other hand, Trasentin (Glick, 1942) and Trasentin 6H and Lachesine (Blaschko, Chou, and Wajda, 1947) have been reported to be unaffected by atropinesterase, and it would be possible, therefore, to compare these compounds on atropine-resistant rabbits. Other more recently synthesized atropine-like compounds

do not appear to have been investigated for their susceptibility to destruction by atropinesterase.

The incidence and transmission of atropine resistance in rabbits have been investigated by Sawin and Glick (1943) and others; it seems to be at least partly controlled by hereditary factors. Consequently, it would be expected that for any one strain the atropine-resistance factor would be present to a fairly consistent degree, but that there would be a variation between different strains. About 90% of our "English" rabbits, bred locally, were atropine-sensitive, whereas roughly a similar percentage of various blue "Beveren" and black rabbits obtained from another dealer were resistant. According to Sawin and Glick, there may be a relation between the presence of atropinesterase in the blood and the extent of black pigment in the rabbit's coat.

Several authors testing anti-acetylcholine agents for salivary blockade in rabbits have reported a wide variability in sensitivity to atropine, but none has related this to the presence of an enzymic mechanism for destroying atropine. Brown and Werner (1949), for instance, reported that 0.075 to 0.3 mg./kg. atropine intramuscularly "markedly inhibited salivary secretion in some animals, but had little effect in others." They therefore used a quantal response, and reported that the minimal dose of atropine which would reduce the salivary flow to below 20 ml. in 2 hr. in five out of six rabbits tested was 600 μg./kg. At least some of the animals used by these workers probably possessed atropinesterase in their blood.

Cahen and Tvede (1952), in a quantal assay of salivary blockade in rabbits, showed that the percentage (probit) of rabbits with at least a 50% inhibition of pilocarpine-induced salivation was linearly related to the log dose of atropine. The ED50 for atropine by this method was 62 μg./kg., whereas assessment by graded response in the same 40 animals gave an ED50 of 110 μg./kg. Such a difference between ED50 values might be expected if it is assumed that certain of these 40 rabbits (but less than half of the total) possessed atropinesterase. Thus, a dose of atropine just sufficient to cause a 50% inhibition of salivation in the sensitive animals—giving an ED50 in quantal response—would produce a mean inhibition of considerably less than 50% on the graded response scale, when the almost negligible responses of the atropine-resistant animals are taken into account.

Cahen and Tvede eventually regarded the rabbit as an unsuitable test animal "because of the variability of the individual responses and its unusual tolerance to atropine." We have found, however, that rabbits without atropinesterase are very sensitive to atropine. In the rabbit the dose of atropine causing a 50% reduction in pilocarpine-induced salivation (3–7 μg./kg. s.c.) is considerably less than that needed in the mouse for a 50% maximal increase in pupil size (100 μg./kg. s.c.) and similar to that causing a 50% inhibition of the hypotensive response to a just-maximal dose of acetylcholine in the cat (1 μg./kg. i.v.) (Brown, Quinton, Acred, Bainbridge, and Turner—unpublished observation). Lands *et al.* (1946) also quote a low dose (1 μg./kg. i.v.) for a 70% inhibition of pilocarpine-induced salivation in rabbits.

Certain workers have administered atropine intravenously (Lands *et al.*, 1946; Tripod, 1949; Chen, 1954; Luduena and Lands, 1954). Atropinesterase may not have time seriously to affect the action of atropine given in this way, particularly when, as in the technique of Luduena and Lands, the salivary flow is measured for two periods of only 10 min. each, immediately before and after the injection of atropine. This point was therefore investigated in two sets of eight rabbits. In the first set, composed entirely of atropine-sensitive animals, the mean salivary response in the 10 min. after an intravenous injection of 10 μg./kg. atropine was 6.0±2.0% (S.E.) expressed as a percentage of the amount of saliva obtained in the preceding 10 min. In the other set of animals, all of which possessed atropinesterase in their blood, the corresponding figures were 63.6± 12.3% (S.E.). Pilocarpine nitrate (1 mg./kg.) was given to both groups 15 min. before the atropine. The difference between the mean salivary responses for the two groups is highly significant. It appears therefore that even with intravenous administration of atropine it is still necessary to use atropine-sensitive rabbits, since the atropinesterase affects the activity of a low dose of atropine even within 10 min. of intravenous injection.

In order to minimize variation between different rabbits, drugs were compared in the same animals. By the use of a (2+2) or (2+2+2) design whereby each of a set of eight rabbits received each of two dose-levels of the drugs tested, the relative potency in terms of a standard has been obtained with its limits of error ($P=0.95$). The values for λ (the standard deviation of a single log dose) ranged from 0.20 to 0.40. We found the relative potencies of atropine methyl nitrate and homatropine in terms of atropine to be 2.3 and 0.009 respectively. Issekutz (1917) reported that atropine methyl nitrate was 3 to 4 times, and homatropine about one fifth, as potent as atropine,

whereas according to Luduena and Lands (1954) the methyl nitrate was eight times as potent as atropine on intravenous administration. It is possible that slowness of absorption of the quaternary compound after subcutaneous injection may partly account for the lower potency obtained by us.

We have not found any reports on the activity of tricyclamol, oxyphenonium, and propantheline in antagonizing pilocarpine-induced salivation in rabbits, although Drill (1954) quotes from some unpublished data supplied by Clark and co-workers that "the intrinsic anti-salivary activity (of oxyphenonium) assayed in rabbits is 1.85 times greater than that of atropine." We have found the relative potency of tricyclamol to be 0.62. The approximate relative potencies for oxyphenonium and propantheline, derived by comparing ED50 values from responses in the same rabbits but not at the same times, were 0.9 and 1.1 respectively.

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References

Ammon, R., and Savelsberg, W. (1949). *Ztschr. f. physiol. Chem.*, **284**, 135.
Blaschko, H., Chou, T. C., and Wajda, I. (1947). *Brit. J. Pharmacol.*, **2**, 108.
Brown, B., and Werner, H. (1949). *J. Pharmacol.*, **97**, 157.
—— Thompson, C., Klahm, G., and Werner, H. (1950). *J. Amer. pharm. Ass.*, **39**, 305.
Bülbring, E., and Dawes, G. (1945). *J. Pharmacol.*, **84**, 177.
Cahen, R., and Tvede, K. (1952). Ibid., **105**, 166.
Chen, J. (1954). Ibid., **112**, 64.
Cushny, A. R. (1920). Ibid., **15**, 105.
Drill, V. (1954). *Pharmacology in Medicine*, chapter 42, p. 18. New York: McGraw-Hill.
Fromherz, K. (1933). *Arch. exp. Path. Pharmak.*, **173**, 86.
Glick, D. (1940). *J. biol. Chem.*, **134**, 617.
—— (1942). *J. Amer. chem. Soc.*, **64**, 564.
—— and Glaubach, S. (1941). *J. gen. Physiol.*, **25**, 197.
Graham, J., and Lazarus, S. (1940). *J. Pharmacol.*, **69**, 331.
Hoekstra, J., Tisch, D., Rakieten, N., and Dickison, H. (1954). Ibid., **110**, 55.
Holton, P. (1948). *Brit. J. Pharmacol.*, **3**, 328.
Issekutz, B. (1917). *Ztschr. f. exp. Path.*, **19**, 99.
Lands, A., Nash, V., and Hooper, K. (1946). *J. Pharmacol.*, **86**, 129.
Luduena, F., and Lands, A. (1954). Ibid., **110**, 282.
Sawin, P., and Glick, D. (1943). *Proc. Nat. Acad. Sci., Wash.*, **29**, 55.
Schild, H. O. (1942). *J. Physiol.*, **101**, 115.
Tripod, J. (1949). *Helv. physiol. Acta*, **7**, 135.

Brit. J. Pharmacol. (1957), **12**, 61.

THE RELEASE OF CATECHOL AMINES FROM ISOLATED CHROMAFFIN GRANULES

BY

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Release of catechol amines from the chromaffin granules of the bovine suprarenal medulla has been studied. Aliphatic monoamines and diamines released catechol amines from chromaffin granules under conditions similar to those under which they are known to promote the release of histamine from suspensions of granules. Carbachol and histamine did not release catechol amines from granules.

Recent work has shown that the pharmacologically active amines are stored in cytoplasmic granules. However, little is known about the mechanism of release from these granules. In his study of the intracellular localization of histamine, Hagen (1954) found that the amine present in a fresh suspension of dog liver granules in isotonic sucrose did not exert its effect upon the blood pressure of the decerebrate cat, but that it was immediately and fully active upon treatment of the suspension with a histamine releaser, *n*-octylamine. Other substances have since also been found to release histamine from a suspension of granules (Mongar and Schild, 1954, 1956; Grossberg and Garcia-Arocha, 1954).

Undenatured chromaffin granules, when injected into the spinal cat, do not immediately exert the full pressor effect which would correspond to their catecol amine content (Blaschko, Hagen, and Welch, 1955). Some time ago, Feldberg (1940) had shown that lysolecithin released adrenaline from "cell debris," and Hillarp and Nilson (1954) have since found that not only lysolecithin but a number of surface-active agents release catechol amines from suspensions of chromaffin granules into the surrounding fluid.

In the present work, the action of a number of compounds, known to be releasers of histamine, was studied. Observations were also made on the action of substances known to release catechol amines from intact chromaffin cells *in situ*.

These results were communicated to the British Pharmacological Society in January, 1956.

METHODS

Preparation of the Large Granule Fraction.—Ox suprarenal glands were collected on ice from the slaughter-house; usually four glands were dissected and the chopped medullae, about 7 to 10 g., were dispersed in a glass homogenizer in 0.3 M-sucrose. The final homogenate was brought to a volume of 50 ml. and then centrifuged for 10 min. at 2,000 rev./min. (950 *g*) in a refrigerated MSE centrifuge. The supernatant fluid, usually 40 to 43 ml., was collected; the sediment, which contained coarse material, red cells, and nuclei, was discarded. The supernatant fluid was again centrifuged, this time in the high-speed attachment of the MSE centrifuge at 2,500 × 4.75 rev./min. (11,000 *g*) for 20 min. At the end of the run the supernatant fluid was decanted and discarded. The inner walls of the centrifuge tubes were wiped dry with tissue-paper and the sediments resuspended by adding 0.3 M-sucrose, so as to bring the suspension up to the original volume. The sediments were thoroughly stirred and the suspension obtained was collected and shaken. This suspension was again centrifuged, in order to reduce the amounts of amine in free solution, this time at 2,800 × 4.75 rev./min. (14,000 *g*) for 20 min. The supernatant fluid was discarded and the sediment resuspended in 0.3 M-sucrose as before. The final volume of the suspension was chosen so that 1 g. of medulla corresponded to 2 ml. of suspension. In the following, this suspension will be referred to as the "large-granule fraction"; it is known that this fraction, which contains the greater part of the catechol amines, is not homogeneous, but can be further subdivided into "top" and "bottom" layers (Blaschko, Hagen, and Welch, 1955).

All centrifugations were carried out at 0° C., and during the preparation all manipulations were carried out as near 0° C. as possible.

Incubation.—The large-granule fraction was incubated under various conditions in order to study the release of catechol amines. Centrifuge tubes of 7 ml. capacity were used; the volume of the final suspension was usually 5.0 ml. This volume included 1.0 ml. of the large-granule fraction and 1.0 ml. of phosphate buffer of *p*H 7.0 (Clark, 1928). (A buffer of appro-

priate *p*H was used in those experiments in which the effect of *p*H was studied.) The total volume also included the substance to be tested as a releaser, dissolved or suspended in 0.3 M-sucrose ; the final volume was brought up to 5.0 ml. with 0.3 M-sucrose. Where necessary, the substances to be tested were neutralized.

Unless stated otherwise, incubation was for 30 min. in a water-bath at 37.5° C. ; the tubes were agitated during incubation. They were at once transferred to an ice-water bath, and then centrifuged at a speed of 3,000 to 4,000 × 4.75 rev./min. (16,000 to 28,000 *g*) for 30 min. The supernatant fluids, which were clear, were collected for the determination of their catechol amine content.

Determination of Catechol Amines.—For this purpose, 1 ml. of the suspension was used ; 1.0 ml. of N-HCl was added and the mixture was frozen. It was stored in the refrigerator at −12° C. After thawing, 0.5 ml. of the mixture was added dropwise to 4.5 ml. of cold ethanol ; this was allowed to stand in the cold for ½ to 1 hr., and was then centrifuged at 450 *g* for 10 min. ; the supernatant fluid from this centrifugation was used for the determination.

The catechol amines released were determined directly in the supernatant fluids after high-speed centrifugation. The method was essentially that of v. Euler and Hamberg (1949), in which the intensity of the colour produced when catechol amines are oxidized by adding iodine is measured. The observation that at *p*H 6.0 adrenaline and noradrenaline contribute equally to the development of colour was confirmed ; the determinations were therefore carried out at *p*H 6.0.

After addition of the iodine the samples were allowed to stand for 3 min. ; sodium thiosulphate was then added to remove the excess iodine, and the colour intensity was read immediately at 529 mμ in a Unicam spectrophotometer.

Substances Used.—The aliphatic diamines were kindly given by Dr. E. J. Zaimis, and Dr. R. Charlier, of Labaz Laboratories, Brussels, supplied compound L–1935 ; the other substances were obtained from commercial sources.

Results

The Effect of Temperature upon Release

When a suspension of the large-granule fraction was incubated, the amount of amine released depended upon the temperature at which the incubation was carried out. In the experiment shown in Fig. 1, incubation was at 0°, 25° and at 37.5° C.; samples were removed at different intervals and centrifuged. Fig. 1 shows the amount of catechol amine found in the supernatant fluids. In this and subsequent experiments, about 10% of catechol amines was present in the supernatant fluid at zero time of incubation. This was probably amine not completely removed when the granules were washed.

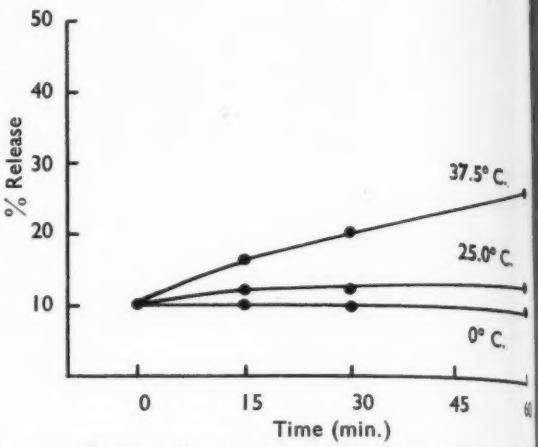

FIG. 1.—The effect of temperature upon the release of catechol amines from chromaffin granules suspended in 0.3M-sucrose at *p*H 7.0. Abscissa: time in min. Ordinate: % of catechol amines released. Temperatures: 0°, 25°, and 37.5° C.

Upon incubation at 0° C., no release of amines occurred and at 25° C. the amounts released were small. At 37.5° C., release was more marked: of the amines present in the granules at the beginning of the incubation (90% of the total amine present in the sample) 18%, or one-fifth, were released during one hour at 37.5° C.

Homogenates in Saline Media

A few comparisons were made of amine release when the medullary tissue was homogenized in the sucrose medium or in a saline medium. In these experiments, the chopped medullary tissue was divided into two portions, one of which was homogenized in 0.3 M-sucrose, the other in the saline medium. Two different saline media were tested in different experiments: one was a mixture of nine parts of 0.15M-NaCl and one part of 0.067M-sodium phosphate buffer of *p*H 7.4. The other medium contained nine parts of 0.15M-KCl and one part of 0.067M-potassium phosphate buffer of *p*H 7.4. Four experiments were carried out, two with the sodium chloride and two with the potassium chloride medium respectively. There was no evidence that homogenizing in an electrolyte medium favoured release. However, there was some evidence that, upon incubation of a large-granule fraction, release was favoured in a saline medium.

The Action of Releasers

Aliphatic Monoamines.—The substances tested included the straight-chain aliphatic monoamines of the series $CH_3(CH_2)_{n-1}NH_2$. All the members of the series from $n=3$ to $n=10$ were available, as well as the amines with 12 and 18 carbon atoms.

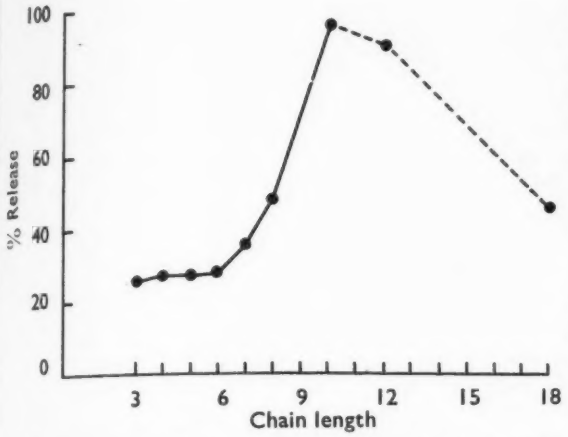

FIG. 2.—Release of catechol amines by aliphatic monoamines of the series $CH_3(CH_2)_{n-1}NH_2$. Abscissa: number of carbon atoms. Ordinate: % release. Incubation at 37.5° C. and *p*H 7.0 in 0.3M-sucrose for 30 min. Amine concentration: 0.97×10^{-3}M.

The results of experiments, in which the amines were tested in a concentration of 0.97×10^{-3}M, are shown in Fig. 2. With increasing length of the polymethylene chain, there occurred an increase in the amount of catechol amines released; the release was maximal for *n*-decylamine. Doubling the concentration of the releasing agents increased the amounts of catechol amines released.

Octadecylamine ($n=18$) did not give a clear aqueous solution in the concentrations used. On incubation with both octadecylamine and dodecylamine the granules appeared to coagulate with the formation of a flocculent precipitate.

Aliphatic Diamines.—Of the series of straight-chain diamines, $NH_2(CH_2)_nNH_2$, the members from $n=3$ to $n=15$ were tested in a concentration of 0.52×10^{-3}M and 1.04×10^{-3}M.

Release of catechol amines by the aliphatic diamines was less than that observed in the presence of the corresponding monoamines of the same chain length in a similar concentration. The relation between chain length and release in the diamine series is shown in Fig. 3; release increased with increasing length of the polymethylene chain, up to the member with 15 methylene groups.

Other Compounds.—Two surface-active substances were tested: saponin and sodium taurocholate. With sodium taurocholate a concentration of 1 mg./ml. was required to release 50% of the catechol amines into the supernatant fluid. For saponin, the corresponding concentration was about 0.2 mg./ml.

The compound L–1935 was tested in a concentration range of 0.1 to 1.0 mg./ml. About 50% of the catechol amines were found in the supernatant fluid after incubation with 0.7 mg./ml. of L–1935. This substance has a brown colour, and it was noted that upon centrifugation the supernatant fluid had become colourless, whereas the sedimented granules were dark brown, in contrast to their normal tan colour; this indicated that the compound L–1935 had been absorbed by the material of the granules.

Carbachol and histamine were tested, as these two substances are known to release adrenaline from the adrenal medulla *in situ*. No release of amines was observed with concentrations from 0.1 to 1.0 mg./ml.

Propamidine was tested in one experiment at a concentration of 0.5 mg./ml.; it was almost without releasing activity.

p*H and Release of Catechol Amines*

Effect upon Spontaneous Release.—When incubation was carried out at 20° C. there was no marked difference in the release of amines between *p*H 5.0 and 8.0. At 30° C., however, there was a significantly higher release at *p*H 5.0, whereas there was little difference at *p*H 6.0, 7.0 and 8.0. This latter result is in general agreement with findings by Hillarp and Nilson (1954).

Effect upon Chemical Release.—Some of the releasing agents tested were incubated with the large-granule fraction at different *p*H and the release of catechol amines was determined. In these experiments the McIlvaine buffer of *p*H 7.0

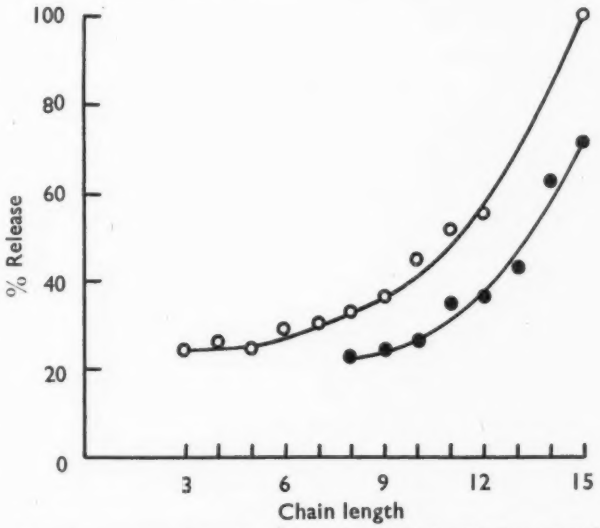

FIG. 3.—Release of catechol amines by aliphatic diamines of the series $NH_2(CH_2)_nNH_2$. Abscissa: number (*n*) of methylene groups. Ordinate: % release. Incubation as in Fig. 2. ●——● Amine concentration 0.52×10^{-3}M; ○——○ Amine concentration 1.04×10^{-3}M.

FIG. 4.—The effect of *p*H upon the release of catechol amines from chromaffin granules by 1: 10-diaminodecane (○——○) and L–1935 (●——●). Abscissa: *p*H. Ordinate: % release. Incubation at 37.5° C. for 30 min.

was replaced by the same amount of McIlvaine buffer of different *p*H (Clark, 1928).

In one experiment the large-granule fraction was incubated with *n*-octylamine in a concentration of 1 mg./ml. at 20° C. for 15 min. Upon centrifugation after incubation, the release of catechol amines was greater as the *p*H increased. Thus the amounts at *p*H 5.0, 7.0, and 8.0 were 23%, 71%, and 87% respectively.

In the experiments shown in Fig. 4, incubation was carried out at 37.5° C. and for 30 min.; the *p*H was 5.0, 6.0, 7.0, and 8.0. The two substances tested were 1 : 10-diaminodecane (0.5 mg./ml.) and L–1935 (0.5 mg./ml.). Fig. 4 shows that with diaminodecane there was a minimum of release at *p*H 6.0 ; at *p*H 5.0, the amount of catechol amines released was slightly greater, and at *p*H 7.0 and 8.0 there was a marked increase in the amounts released. With L–1935, the release at *p*H 5.0 was about the same as at *p*H 6 ; but here, too, the amounts released at *p*H 7.0 and 8.0 were greater.

DISCUSSION

The release of catechol amines from the intact adrenal medullary cell is usually mediated by nerve stimulation. The secretory nerve supply to the gland is a cholinergic one. That carbachol releases adrenaline from the adrenal medulla in the intact animal was demonstrated by Feldberg (1932). In the experiments here reported carbachol proved ineffective as a releaser of catechol amines from chromaffin granules. Another substance which mobilizes the amines from the intact gland *in situ* is histamine (Burn and Dale, 1926) ; histamine also was inactive as a releaser. These observations suggest that the action of choline esters and of histamine upon the chromaffin cell is an action upon the cell membrane. The nature of this effect upon the cell membrane is not known ; possibly it consists in an increased permeability of the membrane, which allows amines present in the cytoplasmic sap to leave the cell and to enter the surrounding tissue fluids and blood. There seems to be no direct effect of these substances upon the storage granules.

A number of other substances were found which release catechol amines from isolated granules. Some of these substances, such as the aliphatic mono- and di-amines, appear to be as active releasers of catechol amines as they are of histamine. That the mechanism of catechol amine release is similar to the release of histamine is further supported by the observation that compound L–1935, which also releases catechol amines, is also known to be an active histamine releaser (Feldberg and Lecomte, 1955). Since these observations were made it has also been shown that the histamine releaser 48/80 releases catechol amines from isolated chromaffin granules (Högberg and Hillarp, 1956).

In the series of aliphatic monoamines, the relationship between length of the polymethylene chain and potency as a releasing agent was similar to that described by Mongar and Schild (1953) for their activity in releasing histamine from guinea-pig lung.

Another similarity between the ability of these substances to release histamine on the one hand, and catechol amines on the other, was in the effect of *p*H. An increase in the histamine-releasing activity of these aliphatic amines, with both increasing and decreasing *p*H, was reported to the British Pharmacological Society in January, 1956, by Mongar, who interpreted his observations by assuming that the histamine-carrying granules were surrounded by a membrane, which was permeable to the releasing amine in its unionized form, whereas the releasing effect was exerted within the granule by the ionized amine. A similar mechanism must also be postulated for the chromaffin granules, and it is of interest in this connexion that electron-microscopic investigation of the chromaffin granules has shown that they are in fact surrounded by a membrane (Lever, 1955 ; Sjöstrand and Wetzstein, 1956).

Whereas the mechanism of release of both histamine and catechol amines from isolated granules appears to be similar, it is interesting that in experiments on intact animals the substances tested are mainly active as releasers of histamine. This could be explained by assuming that there exists a difference in the cell membranes of amine-carrying cells. The cells which store histamine, such as the mast cells, may be characterized by a particularly vulnerable cell membrane, which allows the releasing agents to act upon the granules in the intact cell, whereas the granules in the chromaffin cell are less readily accessible. This would explain why the action of these substances upon the chromaffin granules becomes manifest only after the granules have been isolated.

References

Blaschko, H., Hagen, P., and Welch, A. D. (1955). *J. Physiol.*, **129**, 27.

Burn, J. H., and Dale, H. H. (1926). *J. Physiol.*, **71**, 185.

Clark, M. A. (1928). *The Determination of Hydrogen Ions*, 3rd ed., p. 214. London: Ballière, Tindall and Cox.

von Euler, U. S., and Hamberg, U. (1949). *Acta physiol. scand.*, **19**, 74.

Feldberg, W. (1932). *Arch. exp. Path. Pharmak.*, **168**, 287.

—— (1940). *J. Physiol.*, **99**, 104.

—— and Lecomte, J. (1955). *Brit. J. Pharmacol.*, **10**, 254.

Grossberg, A. L., and Garcia-Arocha, H. (1954). *Science*, **120**, 762.

Hagen, P. (1954). *Brit. J. Pharmacol.*, **9**, 100.

Hillarp, N.-A., and Nilson, B. (1954). *Acta physiol. scand.*, **31**, suppl. 113.

Högberg, B., and Hillarp, N.-A. (1956). *Kungl. Fysiografiska Sällskapetsi Lund Förhandl.*, **26**, 11.

Lever, J. D. (1955). *Endocrinol.*, **57**, 621.

Mongar, J. L., and Schild, H. O. (1953). *Brit. J. Pharmacol.*, **8**, 103.

—— —— (1954). *J. Physiol.*, **126**, 44P.

—— —— (1956). Ibid., **131**, 207.

Sjöstrand, F. S., and Wetzstein, R. (1956). *Experientia*, **12**, 196.

Brit. J. Pharmacol. (1957), **12**, 66.

THE ANTIVIRAL ACTION OF *THREO*-β-PHENYLSERINE

BY

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L-*threo*-phenylserine and esters of *threo*-phenylserine were the most active of a series of compounds tested against influenza A virus in tissue culture. Substitution of the β-OH or α-NH_2 group abolished activity. The activity of phenylserine was reversed competitively by phenylalanine. Phenylserine did not act on free virus or on the adsorption of virus to host cells. It prevented virus growth if added during the first half of the latent period.

Phenylalanine appears to be necessary for virus synthesis and can be supplied by phenylalanylglycine or glycylphenylalanine.

Phenylserine had no significant activity against ectromelia infections in mice, even when the amino acid content of the livers had been depleted by starvation.

During studies of possible inhibitors of protein synthesis for virus chemotherapy trials, many amino acid derivatives have been tested against influenza virus in eggs (Dickinson, 1955). Tissue culture tests have been done in parallel and β-phenylserine was found to be active. This compound inhibits the growth of some bacteria, its action being reversed by phenylalanine (Bergmann, Sicher, and Volcani, 1953; Simmonds, Dowling, and Stone, 1954; Fox and Warner, 1954); recently the antibacterial action of *p*-fluorophenylserine has been reported (Edmonds, Volkman and Beerstecher, 1956). There have been no reports of its antiviral activity, but certain other amino acid analogues have shown antiviral action in tissue culture (Ackerman, 1951; Ackerman and Maassab, 1954; Ackerman, Rabson, and Kurtz, 1954). Matthews and Smith (1955) and Hurst and Hull (1956) include amino acid antagonists in their reviews on "virus chemotherapy."

Phenylserine derivatives are of particular interest in view of the structure of chloramphenicol, which is active against the large viruses of the psittacosis group. This paper reports the inhibition of influenza virus by *threo*-β-phenylserine and various related compounds. The mode of action of phenylserine and its use as a tool in studies of phenylalanine metabolism of virus-infected cells are also described.

METHODS

Virus Strains.—The following influenza A virus strains were kept at −65° C. as allantoic fluids: PR8, NWS, and the Francis and Moore (1940) neurotropic variant of WS (here called WSNF), supplied by Dr. D. A. J. Tyrrell as more suitable for cytopathogenicity tests than NWS. Mumps (Hampstead), vaccinia (Salaman), and ectromelia (Hampstead) were also used. For work *in vivo* ectromelia (Hampstead), mouse-adapted influenza A/PR8 and encephalomyocarditis (EMC) were used. These were kept as freeze-dried, crude suspensions, of known high titre, in 0.5 ml. or 1 ml. volumes. Broth was a satisfactory medium for drying influenza or ectromelia, but EMC was dried as a mouse-brain homogenate suspended in homogenized yolk sacs from chick embryos: this suspending agent was much superior to broth. All three freeze-dried viruses were reconstituted and diluted in broth for testing.

Titration of Viruses.—Serial ten-fold dilutions were titrated in mice or eggs; the inoculum was 0.2 ml. except for intranasal and intracerebral titrations, when it was 0.05 ml. Results were expressed as log LD50 or, for influenza virus titrations in eggs, as EID50 (egg infective dose 50), using the method of Reed and Muench (1938). For influenza virus 9-day chick embryos were infected via the allantoic sac, and the presence of haemagglutinin at 48 hr. was determined by a modified Salk (1944) method. Haemagglutinin titrations were done in plastic blocks, using two-fold dilutions in saline and a final fowl red blood cell concentration of 0.25%; results were expressed as reciprocals of the end-point dilution.

Suspended Fragment Culture and Testing of Drugs against Influenza A/PR8 Virus.—Finely chopped chorioallantoic membrane (CAM) fragments of 9 to 10 day chick embryos were suspended in the maintenance medium, consisting of Earle's saline (Earle, 1943) 2 parts, embryo extract 1 part. The propor-

tions were 0.25 ml. tissue suspension to 2.0 ml. medium, in tightly bunged 25 ml. flasks; one membrane provided tissue for 6 flasks. Then 0.25 ml. virus suspension containing 10^4 EID50 was added, and, 1 hr. later, 0.5 ml. of the drug (serial five-fold dilutions); controls were made up to the same volume. After 48 hr. stationary incubation at 37° C. the haemagglutination titres of the supernatants were found, and the possibility of false haemagglutination or haemagglutination inhibition was checked. Three flasks for each drug dilution and three controls were used throughout; haemagglutinin titres of controls were always 64–256. To find the effect of the drug on the cells, tissue fragments were removed after 24 hr., washed and embedded in plasma, then rotated in growth medium (2 parts horse serum, 3 parts maintenance medium) for 48 hr.; outgrowths of cells were observed microscopically from 24 hr. Results were expressed both as the drug concentration inhibiting the growth of virus and also as a ratio of the cytocidal concentration to the virus inhibitory concentration. To determine the cytostatic concentration a similar method was used, the drug being present during rotation of the embedded fragments.

Compounds.—Many of the compounds tested occur as optical isomers and also as *threo* and *erythro* forms. Except where otherwise stated, the racemic form is implied. Phenylserine is the DL-*threo* form unless otherwise stated. All the compounds tested, with the exception of 4550, 4923, and 4385 (Nicholson, see appendix), and the mandelic acid amidinium chlorides (Bristow, personal communication), have been described in the literature.

Solutions were prepared at *p*H 7.4 and kept frozen at −20° C. between experiments. Several, such as phenylserine itself, decomposed in solution, especially on heating. Solutions were not sterilized, but 100 i.u. each of penicillin and streptomycin were added per ml. tissue culture medium to inhibit bacterial growth.

Growing Cell Cultures.—The method of Tyrrell (1955) was used to "trypsinize" lungs of 9 to 14 day chick embryos. One ml. cell suspension, containing approximately 10^7 cells/ml. in growth medium, was allowed to settle overnight in horizontal, tightly stoppered, 150 by 13 mm. tubes. In some experiments 40 by 14 mm. vials were used; 0.5 ml. cell suspension was used in these. After removal of the supernatant, virus and drug dilutions were added and the tubes rotated at 12 r.p.h. The cells were examined microscopically daily and haemagglutination titres determined at 48 and 72 hr. The fluid added before rotation was either maintenance medium, BAF medium (2 parts 1% Armour's bovine serum albumin fraction V, 3 parts maintenance medium) or CWB medium, a synthetic medium (Waymouth, 1955) used here for the studies on phenylalanine metabolism.

Tests in Eggs.—The method of Dickinson (1955) was used, both drug and virus being given by the allantoic sac. Four- or five-fold dilutions of the drug were tested, up to and including the toxic dose (or 40 mg./egg if not toxic at this dose).

Tests in Mice.—White mice (20 g.) in groups of 10 to 30 were used. The dose of virus was calculated to infect all and to kill at least half of the mice of the control groups; controls were always infected last. Subcutaneous treatment commenced the day before infection and was continued at the maximum tolerated dose until the experiment ended. Influenza A/PR8 was given intranasally, influenza A/NWS intracerebrally, ectromelia and EMC intraperitoneally. Results were assessed on average survival times, and, for influenza A/PR8, on the extent of lung lesions; they were assessed for significance by the "t" test.

Phenylalanine Assay.—*Escherichia coli* K12/58–278 was kindly supplied by Dr. B. D. Davis. This mutant was inhibited by valine, but a valine-resistant mutant was readily obtained and was much more satisfactory for assay purposes than *Leuconostoc mesenteroides* previously used. The medium consisted of lactic acid 2.4 g., NaCl 5 g., $(NH_4)_2HPO_4$ 1 g., KH_2PO_4 1 g., $MgSO_4.7H_2O$ 0.2 g., and distilled water to 1 litre. The *p*H was adjusted to 7.0 and the medium was autoclaved at 15 lb./15 min. Biotin (0.25 μg./l.) was added and 3 ml. volumes were distributed. One ml. of dilutions of standards (range 0.01 to 10 μg./ml. phenylalanine) and unknowns were then added; tubes were prepared in triplicate and all dilutions made in the medium. The inoculum was prepared from a 24 hr. culture in the above medium containing 100 μg./ml. phenylalanine. After three washings in saline the final deposit was made up to the original volume in the medium and then diluted 1 in 10; one drop of this dilution was added to each tube, and the cultures were incubated at 37° C. for 48 to 72 hr. Growth could be detected at a concentration of 0.1 μg. phenylalanine/tube.

Results

Activity of Phenylserine.—Phenylserine, at 0.1 to 0.2 mg./ml., inhibited the production of influenza virus, as judged by haemagglutination, in the suspended fragment test. It was not cytocidal to CAM fragments at 10 mg./ml. The haemagglutinin titres at 48 hr. for treated cultures were all <2, the controls being 64–256. These results were checked by infectivity determinations in eggs; the control titres at 48 hr. were 7.27, the initial titre being 1.75. The titres at 48 hr. for phenylserine-treated cultures were 1.75 (1.0 mg./ml.) and 5.75 (0.25 mg./ml.). The methyl ester of phenylserine was more active, virus increase being completely inhibited at 0.2 mg./ml., judged by both haemagglutination and infectivity determinations.

Toxicity of Phenylserine to Growing Chick Embryo Cells.—Explants of CAM tissue were grown in growth medium in roller tubes. Cell growth occurred at 1.0 mg./ml. phenylserine. Similar results were found for lung tissue explants

and trypsinized lung cells. When the cells were infected with influenza virus in the same medium, phenylserine prevented virus growth (haemagglutination test) at 0.2 mg./ml. Thus phenylserine prevented virus growth in both suspended fragments and in growing cells, but the cytostatic concentration was lower than the cytocidal concentration, as might be expected.

Mode of Action of Phenylserine

To determine whether phenylserine was acting on extracellular virus, on adsorption of virus or during the multiplication of virus within the cell, the following experiments were done. Influenza A/PR8 in maintenance medium and 0.2 mg./ml. phenylserine were used, unless otherwise stated.

Inactivation of Extracellular Virus.—The rates of inactivation of influenza virus in the absence of cells with and without phenylserine (0.2 mg./ml.) were found by finding the EID50 at 0, 6, 24 and 48 hr. There was no significant difference between the two inactivation rates. The initial titre was 5.75; titres at 48 hr. were 3.5 (controls) and 3.75 (phenylserine treated).

Action on Adsorption of Virus and on Various Stages in the Growth Cycle.—To find the mode of action of an antiviral drug, the best system has uniform single cells and a virus which can be assayed accurately. This is easy with bacterial viruses and would be possible with the cytopathogenic western equine encephalomyelitis virus and HeLa cells (Dulbecco and Vogt, 1954). A simpler, if less accurate, method for influenza virus is to use the suspended fragment technique, infect the cells very heavily and remove the unadsorbed virus by washing and antiserum treatment; virus release can be followed by infectivity or haemagglutinin titrations. In this system the cell population is not uniform and virus release is spread over a long period of time. However, the main features of the growth cycle are clear—a latent period followed by exponential release of virus, rising to a constant value after several hours.

In these experiments a total time of 1 hr. was allowed for adsorption and washing; quickly adsorbed virus had, therefore, possibly an hour's start on that adsorbed towards the end of the period. Cultures were prepared as in the standard CAM fragment test, but a heavier inoculum (0.25 ml. of a 1/25 dilution of stock allantoic fluid) was used. After a 50 min. adsorption period the excess virus was removed by three washings, the second one incorporating specific antiserum. The washing took 10 min., so that zero time was at 60 min. Phenylserine either was present during the adsorption period (and removed during the washing procedure), or was added at intervals after zero time. Infectivity determinations were made on the supernatants at zero time and at 8½ hr., by which time virus was known to be released from control cultures. Table I shows the results of these tests. Phenylserine had no effect when present only during the adsorption period. If added up to 4 hr. after the end of the adsorption period it prevented virus growth. Tests where the drug was added at more frequent intervals, but using haemagglutination as the index of virus production, confirmed that phenylserine acted during the first half of the latent period. In these tests the inoculum was 0.25 ml. of a 1/2.5 dilution of allantoic fluid and the phenylserine concentration was 0.5 mg./ml. The tests were carried out in rotating flasks and

TABLE I

ACTION OF PHENYLSERINE (0·2 MG./ML.) ADDED AT DIFFERENT TIMES DURING THE GROWTH OF INFLUENZA A VIRUS IN TISSUE CULTURE

Virus titre at time 0 (end of adsorption period of 1 hr.)=1.5.

Phenylserine Treatment	Log Infectivity Titre (EID50)	
	8½ hr.	24 hr.
Present during adsorption period (−1 to 0 hr.) ..	3·5	
Added at 0 hr.	1·5	<1·0
1 ,, ...	1·5	
2 ,, ...	1·25	
4 ,, ...	1·5	
7½ ,, ...	3·5	
Control (no phenylserine) ..	3·25	>4·0

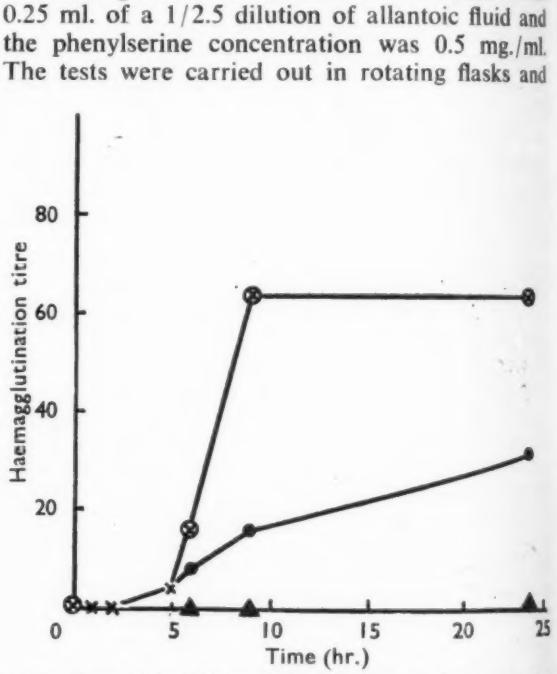


FIG. 1.—Effect of the addition of phenylserine at varying times during the growth cycle of influenza virus. ▲——▲ added at 0, 1, or 2 hr. ●——● added at 4 hr. O——O added at 5 hr. X——X Control.

haemagglutinin production was detectable at 5 hr. (somewhat earlier than in stationary cultures); Fig. 1 shows the result of a typical experiment. It was not possible to give a critical "time of action" from this type of test, especially as there was some, but not complete, inhibition of haemagglutinin production when phenylserine was added, even after halfway through the latent period. The possibility that phenylserine interfered with the release of virus from the cells was checked by grinding up the cells; treated cells contained no virus, whereas controls contained large amounts.

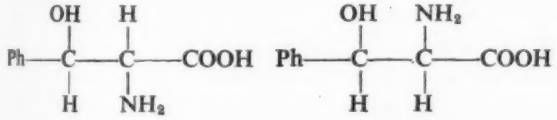
Action on Cytopathogenicity of Influenza A/WSNF on Lung Tissue.—Since phenylserine prevented virus growth even from large inocula of strain PR8, its action against large inocula of strain WSNF, which destroys chick embryo lung cells (Tyrrell, 1955), was investigated. At 1.0 mg./ml. it prevented both the development of haemagglutinin and the destruction of the cells by the virus; when 1 mg./ml. phenylalanine was added to antagonize the action of phenylserine, haemagglutinin production and the cytopathogenic effect occurred as in controls.

Use of Phenylserine in Virus Studies

The antiviral action of phenylserine was used in two ways: (1) As a starting point to find compounds of greater activity and to relate activity to structure; (2) as a tool to investigate the metabolism of virus infected cells, in the first place by antagonism studies.

Structure and Activity.—The relevant compounds prepared and tested, and their activities, appear in Table II.

There are four isomers of phenylserine—i.e., the D and L forms of the *threo* and *erythro* configurations:



The *threo* configuration is that of chloramphenicol, which has the structure indicated in Table II and which is, of course, only active against the largest viruses. Against influenza in these experiments, it was only active at 1.6 mg./ml., whereas DL-phenylserine was active at 0.1 mg./ml. The activity of phenylserine was confined to the L form of the *threo* isomer. The D form was inactive, but did not interfere with the action of the L form, and all work on further variation of the molecule refers

TABLE II

ACTIVITY OF PHENYLSERINE AND RELATED COMPOUNDS AGAINST INFLUENZA A IN TISSUE CULTURE

Bz=Benzyl. p=Partial activity. ..=Not found if compound was inactive at 1 mg./ml., insoluble, or not available in adequate amount. * Configuration unknown.

Ref. No.	Structure	Conc. Inhibiting Virus (mg./ml.)	Therap. Ratio
1412	DL-*threo*-phenylserine	0·1	100
5012	D- ,, ,,	>1·0	..
5223	L- ,, ,,	0·04p	..
4233	DL-*threo*-(*p*-hydroxyphenyl)serine	>1·6	..
4058	DL-*erythro*-phenylserine	>1·6	..
	DL-threo-*isomers of* Ph.CHOR¹.CHNHR².COR³		
	R¹ R² R³		
4386	H H OMe	0·04	>250
4536	H H OEt	0·04p	≈250
4549	H H OBu	0·04	>250
5024	H H OC_6H_{11}	0·04p	≈25
4550	H H $NHNH_2$	0·2p	..
4923	H H NHOH	0·2	5
4599	Me H OH	>1·0	..
4387	CO.Me =CHPh OH	1·0p	..
4433	H COMe OH	>1·0	..
5082	H $COCHCl_2$ OH	>1·0	..
5079	H $COCH_2Cl$ OH	1·0	1
Peptide	H $COCH_2NH_2$ OH	0·2p	..
	Threo-*isomers* (*except* 4540) *of* Ph.CHOH.CHNHR.CH_2OH		
4540	*Erythro*-phenylserinol; R=H	0·2p	..
4539	*Threo*-phenylserinol R=H	1·0	..
1796	*p*-NO_2 ,, ,, R=H	1·0	1
1272	*m*-NO_2 ,, ,, R=H	1·0	5
1237	*p*-Cl ,, ,, R=H	1·0	1
1273	*m*-NO_2 ,, ,, R=$COCHCl_2$	>1·0	..
1155	*m*-NO_2 ,, ,, R=COMe	>1·0	..
1161	,, ,, R=COMe	>1·0	..
	p-NO_2 ,, ,, R=$COCHCl_2$ (Chloramphenicol)	1·6	..
	Mandelamidine hydrochlorides Ph.CHOH.C(=NH)(NHR).HCl		
1053	R=H	1·0	..
1102	*m*-OH R=Me	>1·0	..
1055	*m*-OMe R=H	1·0p	..
1068	*m*-OH R=H	>1·0	..
1071	3:4(OH)$_2$ R=H	1·0p	..
1175	*m*-Cl R=H	1·0	1
1176	*p*-Cl R=H	1·0	1
1180	*m*-OBz R=H	0·2	5
1177	*m*-OBz R=$CHMe_2$	0·2	5
1178	*m*-OBz R=C_7H_{15}	0·04	1
1179	*m*-OBz R=Cyclohexyl	0·04p	≈5
1282	*m*-OBz R=$CH_2.CH_2.NEt_2$	1·0p	..
1128	PhCHOH.C(=N—CH_2)(NH—CH_2)	1·0p	..
1205	*m*-OBz derivative of 1128	0·2	5
1206	*p*-Cl derivative of 1128 (double compound with ethylenediamine)	1·0p	..
	Phenylalanyl derivatives		
.	DL-Phenylalanine	>5·0	..
.	D-Phenylalanine	>1·0	..
.	L-Phenylalanine	>1·0	..
1435	DL-N-Acetylphenylalanine	>1·0	..
5078	DL-Phenylalanine ethyl ester	1·0p	..
.	D(+)-3:4-Dihydroxyphenylalanine	>1·0	..
.	L(−)-3:4-Dihydroxyphenylalanine	>1·0	..
4967	DL-Phenylalanylglycine	>1·0	..
5023	Glycyl-DL-phenylalanine	>1·0	..
4474	Poly-DL-phenylalanine	>0·2	..

TABLE II—*continued*

Ref. No.	Structure	Conc. Inhibiting Virus (mg./ml.)	Therap. Ratio
Miscellaneous compounds			
.	DL-*threo*-Threonine	2·0p	..
.	DL-Serine	5·0	..
.	β-Hydroxyglutamic acid*	1·0p	..
4966	(2-Furyl)-CHOH.CHNH$_2$.COOH*	>1·0	..
4385	Ph.CH — CH COOH O NH CO CH$_2$OH	>1·0	..
1209	Ph—C—NO$_2$ CH$_2$OH	0·04	5

to the DL form. In assessing results, activity in the tissue culture tests was only considered of interest if below 1 mg./ml. and egg tests were done where supplies permitted.

For activity in tissue culture, Table II shows that the NH_2- and OH-groups had to be free but that the COOH-group could be modified, though with no gain in activity except in the case of the esters. Omission of the ring or ring substitution with *p*-OH also prevented activity. The fact that glycylphenylserine was active, in spite of the N-substitution of phenylserine, suggests that it was broken down to phenylserine. Unfortunately phenylserylglycine was not available. Since both the β-OH and α-NH_2 appeared to be necessary for activity, it was not surprising that the phenylalanyl derivatives tested were inactive.

Nothing more active, or with a better therapeutic ratio, than L-phenylserine or the DL esters was found, and, of the series of esters tested, the methyl was considered the best because of the results of the egg tests. Although all the esters had therapeutic ratios of about 250 in tissue culture, the methyl ester was active at 40 mg./egg whereas the other esters were toxic at this dose and inactive at lower doses. The relative inactivity in eggs was probably due to the presence of phenylalanine which antagonized the action of both phenylserine and its ester (see later). Because of the ease of preparation compared to L-phenylserine itself, DL-phenylserine methyl ester was used for *in vivo* tests.

Various mandelic acid amidinium chlorides were also tested, but, though several were active, the activity was associated with toxicity and the therapeutic ratios were very poor. The action of compounds 1177 and 1180 (Table II), unlike that of phenylserine and phenylserine methyl ester, was not reversed by phenylalanine.

The Use of Phenylserine in Studying Phenylalanine Utilization

Antagonism Experiments. — The following amino acids: DL-serine, glycine, L-tyrosine, L-histidine, L-glutamic acid, DL-valine, DL-alanine, DL-lysine, DL-tryptophane, and L-, D-, and DL-phenylalanine, were tested for their effect on the antiviral action of phenylserine in the suspended fragment test. At concentrations of 1 mg./ml. of both phenylserine and amino acid, only L- and DL-phenylalanine had any antagonistic effect; D-phenylalanine had none. In further work, when the concentrations of phenylserine and phenylalanine were varied, the DL-isomer was used. Phenylalanine itself did not inhibit the growth of virus at 5 mg./ml. and its action in antagonizing the action of phenylserine was competitive in nature. Phenylserine was antagonized by an equal amount of phenylalanine over a range of concentrations of both from 0.2–5.0 mg./ml. Phenylalanine also antagonized competitively the action of phenylserine methyl ester and glycylphenylserine.

The reversal by phenylalanine of the phenylserine inhibition of virus growth was also tested in sheets of chick embryo lung tissue. Cells were grown in growth medium, infected by influenza A/PR8 and then maintained in either maintenance or CWB medium. Phenylalanine antagonized the action of phenylserine as in the suspended fragment test. These results possibly explain the inactivity of phenylserine in eggs, although the concentration of phenylalanine in the cells of the allantoic sac is not known.

Peptide Utilization.—Eagle (1955a) found that glycylphenylalanine could replace phenylalanine in tissue cultures of HeLa cells and strain L fibroblasts, but he did not determine whether dipeptides were used intact. This is, indeed, difficult to prove, but one approach to the problem is to see whether natural peptides antagonize an antagonist of one of the constituent amino acids.

Using the suspended fragment test, the following compounds were tested at 5:1 and 1:1 ratios against phenylserine: glycylphenylalanine, phenylalanylglycine, glycylglycine, N-acetylphenylalanine and phenylalanine ethyl ester; they were also tested by themselves to rule out any antiviral action of their own. Phenylalanylglycine, glycylphenylalanine and phenylalanine ethyl ester antagonized the action of phenylserine at an approximate ratio of 1:1. Glycylglycine and N-acetylphenylalanine, even at a 5:1 ratio, did not. Since blockage of the NH_2 of phenylalanine prevents its antagonistic effect, it appears unlikely that glycylphenylalanine

is utilized intact. It is possible that phenylalanine ethyl ester is hydrolysed to the free acid which would itself antagonize phenylserine. Solutions of this ester give strongly positive ninhydrin reactions, but one cannot, from the present evidence, eliminate the possibility that phenylalanine compounds (including phenylalanylglycine) with the carboxyl group substituted may antagonize phenylserine. As far as can be judged, since only small amounts of the peptides were available, both phenylalanylglycine and glycylphenylalanine antagonized phenylserine competitively; thus 5 mg. peptide antagonized 5 mg. drug, 1 mg. antagonized 1 mg. drug, 0.2 mg. antagonized 0.2 mg. drug.

Evidence for the utilization of peptides was also sought by using them as sole sources of phenylalanine in sheets of infected and uninfected lung tissue. Cell suspensions of lung tissue were grown in growth medium, which was then replaced by modified CWB media, in which DL-phenylalanine (0.1 mg./ml.) and glycine (0.2 mg./ml.) were replaced by 0.4 mg./ml. phenylalanylglycine or glycylphenylalanine. After 24 hr. rotation, the media were renewed and half the cultures infected with strain PR8. No gross degeneration occurred in either infected or uninfected tissue within 6 days, in either CWB or the modified media. Virus was produced in all media and even, in some experiments, when added free or combined phenylalanine was not present. Presumably sufficient phenylalanine was present within the cells to allow virus growth unless the utilization of phenylalanine was stopped by an antagonist. The amount of free phenylalanine was less than 0.1 μg./vial, as determined by the *E. coli* assay, but it could be detected if the contents of several vials were pooled. In these assays streptomycin was omitted from the medium and the penicillin inactivated during the extraction of the amino acids by boiling. It was not possible to use the *E. coli* assay culture to follow the postulated breakdown of peptides since it utilized the peptides as readily as free phenylalanine.

Apart from the evidence that glycylphenylalanine is broken down, there is also evidence that glycylphenylserine is broken down because the antiviral action of this peptide is antagonized by phenylalanine.

Effect of Phenylserine on Other Viruses

Other virus-cell systems were investigated to see if phenylserine prevented the growth of other viruses and also to form some basis for *in vivo* tests.

Using a similar technique to the influenza test, but with an incubation period of 8 days, it was found that mumps virus was inhibited by phenylserine. Vaccinia and ectromelia viruses (passaged in eggs to give good lesions on the CAM) were also grown in tissue culture, using whole chick embryo fragments and CAM fragments respectively. The viruses were titrated by plaque counts on the CAM of 10–13 day embryos. Phenylserine (1.0 mg./ml.) prevented the 10- to 100-fold rise in infectivity shown by control cultures.

The growth of EMC virus in suspended fragments of brain tissue from one-day-old mice was inhibited by 0.2 mg./ml. phenylserine in BAF medium; toxicity to cells was not determined. Growth of EMC virus was assessed by a haemagglutination test (modified from Gard and Heller, 1951): 0.25 ml. of 0.2% sheep cells in KCl solution (10.43 g./l.) was added to 0.25 ml. of dilutions of the tissue culture supernatants removed after incubation for 1, 2, and 3 days. The diluent was a 1:1 mixture of barbiturate buffer (*p*H 7.2) and KCl solution. Tests were read after 2 hr. at 4° C. Control titres were 8–32 at 2 to 3 days; titres of phenylserine-treated cultures were all less than 2.

Tests in Mice

Mice tolerated a daily subcutaneous dose of 2 mg./g. of phenylserine or its methyl ester and the effective inhibitory concentrations in tissue culture were 0.2 mg./ml. and 0.04 mg./ml. respectively. Phenylalanine in the body would obviously reduce any activity, but small-scale *in vivo* tests were done against viruses which had been inhibited in tissue culture, such as influenza, ectromelia and EMC.

Both phenylserine and its ester, given subcutaneously, were inactive against influenza A given either intranasally (PR8 strain) or intracerebrally (NWS strain). Phenylserine was also inactive against the PR8 strain, given intranasally, when treatment was given as an aerosol (1 hr. daily using a 2.5% solution).

In the first test against EMC virus, results were: controls 0/20, phenylserine methyl ester 6/20 survivors, but in a second test the ester was inactive. The EMC-Col SK group of viruses is not very satisfactory for *in vivo* testing, since the response of control groups to a fixed dose of virus, given intraperitoneally, sometimes varies with different batches of the same strain of mice, even though these are the same weight. This means that *slight* activity may not be observed simply because in that particular experiment the infection is too severe. The work of Hurst, Melvin, and Peters (1952) on other neurotropic infections of mice showed that a weak therapeutic effect could be lost when the infection was more virulent than expected.

It was obvious that the chief hope of antiviral action *in vivo* lay in reducing the phenylalanine level. The brain is the last organ in the body to suffer amino acid depletion, but the liver protein level can be considerably reduced by a 48 hr. fasting period (Addis, Poo and Lew, 1936). It was hoped to time this depletion so that it occurred when a virus susceptible to phenylserine would normally be multiplying there. Ectromelia was a suitable virus and the mice were fasted at different times. Table III shows the results of these

TABLE III

PHENYLSERINE METHYL ESTER TESTED AGAINST ECTROMELIA VIRUS IN MICE

Treatment, indicated by PS ester, was 2 mg./g./daily subcutaneously, from the day before infection. The mice were infected on day 0. Fasting, with or without PS ester treatment, is indicated.

	Treatment (PS Ester and/or 48 Hr. Fast)	No. Survivors (At Time Indicated)	Average Survival Time (Days)
I	Controls	1/15 (10 days)	7·2
	PS ester	1/14 ,,	7·4
	Fasted −2, −1 days	1/13 ,,	7·6
	,, −1, 0 ,,	1/10 ,,	7·1
	,, −1, 0 (+PS ester)	2/14 ,,	8·1
	,, +3, +4 days	4/5 ,,	9·8
II	Controls	0/22 (9 days)	5·6
	Fasted 0, +1 days	1/22 ,,	6·2
	,, +1, +2 ,,	0/22 ,,	5·8
	,, +2, +3 ,,	1/22 ,,	5·8
	,, +3, +4 ,,	5/22 ,,	6·9
	,, +4, +5 ,,	1/22 ,,	6·3
III	Controls	6/28 (15 days)	9·25
	Fasted +3, +4 days	6/28 ,,	9·6
	,, +3, +4 (+PS ester)	5/28 ,,	9·3
	,, +4, +5 days	3/28 ,,	8·9
	,, +4, +5 (+PS ester)	10/28 ,,	10·5

experiments; in none was $P<0.05$ for the difference in survival times. In experiment III, virus was isolated from all control animal livers, but only from 3/10 fasted and treated animals.

DISCUSSION

The results indicate that *threo*-β-phenylserine exerts its effect against influenza virus during the first half of the latent period, presumably by interfering with the utilization of phenylalanine. It is relatively non-toxic to the host cells investigated compared with, for example, *p*-fluorophenylalanine which was stated to be active against poliomyelitis virus at 0.04–0.1 mg./ml. but cytostatic at 0.1 mg./ml. (Ackerman *et al.*, 1954). It is of interest that the active phenylserine has the same *threo* configuration as chloramphenicol, but, whereas chloramphenicol is the D-(−) isomer, the active phenylserine is the L-(−) isomer, the configuration of naturally occurring phenylalanine. The replacement of the amino-group by $NHCOCHCl_2$ abolishes activity against influenza virus, although this radical is present in chloramphenicol. Chloramphenicol is thought to act at a late stage in the protein synthesis of bacteria (Hahn, Wisseman and Hopps, 1954), but its mode of action on the larger viruses has not been determined.

It is not possible to correlate the antibacterial and antiviral activities of the phenylserines. Early workers did not distinguish between the different isomers, and the test organism and medium varied. The phenylalanine-requiring mutant of *E. coli* K12, used in the assays, was found by us to be unaffected by amounts of phenylserine 100 times that of the phenylalanine present, the concentrations of the latter ranging from 1.0 to 100 μg./tube. Many bacteria synthesize their own phenylalanine, but this amino acid is essential for the growth of animal cells in tissue culture (Eagle, 1955b). Although cells remain sufficiently healthy to support virus growth in the absence of added L-phenylalanine, they do not multiply and in time they degenerate.

Conclusions based on the study of analogues must be assessed cautiously, but this work suggests that phenylalanine is necessary for virus production, at least in the system used here, and that it can be supplied as phenylalanylglycine or glycylphenylalanine. The evidence supports the view that glycylphenylalanine is broken down by virus-infected cells, but there is no conclusive evidence for or against the breakdown of phenylalanylglycine, though this seems likely. It is only possible, from the present work, to refer to virus-infected cells since all the metabolism work was interpreted in the light of virus production, as measured by haemagglutination; there is no information about corresponding events in uninfected cultures.

Although in some of the experiments with ectromelia treated and fasted mice survived longer than the controls, the *in vivo* tests were inconclusive and no result was statistically significant. The antagonism of the antiviral action of phenylserine by phenylalanine at a 1 : 1 ratio makes *in vivo* activity unlikely. Furthermore, an enzyme present in the liver destroys phenylserine, although the *erythro* form is attacked much more readily than the *threo* (Shaw and Armstrong, 1955).

APPENDIX

THE PREPARATION OF HYDRAZINE AND HYDROXAMIC ACID OF PHENYLSERINE

By J. S. NICHOLSON

Threo-*Phenylserylhydrazide*. — *Threo*-Phenylserine ethyl ester (2 g.) (Shaw and Fox, 1953) in 10 ml. absolute alcohol and 4 ml. of 90% hydra-

zine hydrate were refluxed for 1½ hr. On cooling to 0° C. the hydrazide crystallized ; it was collected and washed with a small volume of absolute alcohol. Recrystallization of the hydrazide (0.9 g., m.p. 135–137° C.) from absolute alcohol gave colourless needles, m.p. 135.5–137.5° C. (Found: C, 55.5 ; H, 6.7 ; $C_9H_{13}N_3O_2$ requires C, 55.4 ; H, 6.7%.)

Threo-*Phenylserylhydroxamic Acid.*—Hydroxylamine hydrochloride (0.695 g.), dissolved in 0.7 ml. water, was treated with a solution of sodium ethoxide from 0.23 g. sodium in 10 ml. absolute alcohol. The filtrate from sodium chloride was refluxed with 2.1 g. *threo*-phenylserine ethyl ester for 2 hr., cooled and diluted with an equal volume of ether. The solid (0.57 g.) which separated was collected, dried and recrystallized from water ; the yield was 0.2 g., m.p. 160° C. (decomp.). Recrystallization from water gave colourless prisms, m.p. 160° C. (decomp.). (Found: C, 55.3 ; H, 6.5: $C_9H_{12}N_2O_3$ requires C, 55.1 ; H, 6.1%.)

The reaction between *threo-β*-phenylserine and benzyl chloroformate in alkaline solution gave a compound, which is believed to be 4-carboxy-5-phenyloxazolid-2-one, crystallizing from ethyl acetate-chloroform in colourless rods, m.p. 202–203° C. (decomp.). (Found: C, 57.8 ; H, 4.3 ; N, 7.0 ; neutralization equivalent 210: $C_{10}H_9NO_4$ requires C, 58.0 ; H, 4.3 ; N, 6.8% ; neutralization equivalent 207.)

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References

Ackerman, W. W. (1951). *J. exp. Med.*, **93**, 333.
—— and Maassab, H. F. (1954). Ibid., **100**, 329.
—— Rabson, A., and Kurtz, H. (1954). Ibid., **100**, 437.
Addis, T., Poo, L. J., and Lew, W. (1936). *J. biol. Chem.*, **115**, 117.
Bergmann, E. D., Sicher, S., and Volcani, B. E. (1953). *Biochem. J.*, **54**, 1.
Dickinson, L. (1955). *Brit. J. Pharmacol.*, **10**, 56.
Dulbecco, R., and Vogt, M. (1954). *J. exp. Med.*, **99**, 183.
Eagle, H. (1955a). *Proc. Soc. exp. Biol., N.Y.*, **89**, 96.
—— (1955b). *J. exp. Med.*, **102**, 37.
Earle, W. R. (1943). *J. nat. Cancer Inst.*, **4**, 165.
Edmonds, E. J., Volkman, C. M., and Beerstecher, E. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 80.
Fox, S. W., and Warner, C. (1954). *J. biol. Chem.*, **210**, 119.
Francis, T., and Moore, A. E. (1940). *J. exp. Med.*, **72**, 717.
Gard, S., and Heller, L. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 68.
Hahn, F. E., Wisseman, C. L., and Hopps, H. E. (1954). *J. Bact.*, **67**, 674.
Hurst, E. W., Melvin, P., and Peters, J. M. (1952). *Brit. J. Pharmacol.*, **7**, 455, 473.
—— and Hull, R. (1956). *Pharmacol. Rev.*, **8**, 199.
Matthews, R. E. F., and Smith, J. D. (1955). *Advances in Virus Research*, vol. 3, p. 51. New York: Academic Press Inc.
Reed, L. J., and Muench, H. (1938). *Amer. J. Hyg.*, **27**, 493.
Salk, J. E. (1944). *J. Immunol.*, **49**, 87.
Shaw, K. N. F., and Armstrong, M. D. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 673.
—— and Fox, S. W. (1953). *J. Amer. chem. Soc.*, **75**, 3417.
Simmonds, S., Dowling, M. T., and Stone, D. (1954). *J. biol., Chem.*, **208**, 701.
Tyrrell, D. A. J. (1955). *J. Immunol.*, **74**, 293.
Waymouth, C. (1955). *Texas Rep. Biol. Med.*, **13**, 522.

Brit. J. Pharmacol. (1957), **12**, 74.

THE INFLUENCE OF POTASSIUM CONCENTRATION ON THE ACTION OF QUINIDINE AND OF SOME ANTI-MALARIAL SUBSTANCES ON CARDIAC MUSCLE

BY

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The action of quinidine and some other antimalarial substances on cardiac muscle has been shown to be closely related to the K^+ concentration of the surrounding medium. The depression of the amplitude and rate of the contractions of the isolated perfused rabbit heart and of the isolated rabbit atria which the antimalarial substances produced seems to be due to a diminution of the permeability of the membrane to K^+, since it can be reversed by lowering the external K^+ concentration. During exposure to any of the antimalarial compounds tested, the normal inhibitory action of acetylcholine was converted to a stimulant action. This stimulant action of acetylcholine is probably due to its effect in increasing the permeability of the membrane to K^+. There were slight differences in behaviour between proguanil and quinidine on the one hand, and chloroquine, mepacrine and pyrimethamine on the other. The observations may explain the action of quinidine-like substances in abolishing fibrillation.

The experiments to be described in this paper resulted from an observation made while investigating the action of atropine on ventricular fibrillation induced in the isolated rabbit heart. Atropine sulphate perfused through the coronary vessels of the normally beating heart in a concentration of 10^{-6} g./ml. caused a large reduction in the amplitude of beat, but when perfused through the heart in a solution containing one half the normal potassium it had no such effect. Furthermore, the depression produced by atropine when the K^+ concentration was normal could be reversed by changing to a fluid containing half the normal potassium.

Observations were then made to see if the action of quinidine on the isolated perfused heart and on the isolated atria was affected in the same way by reducing the K^+ concentration. A study was also made of the action of chloroquine, mepacrine, proguanil, and pyrimethamine.

The actions of proguanil and quinidine on isolated rabbit atria, particularly their antagonism to acetylcholine, have already been described in detail (Burn and Vane, 1949; Briscoe and Burn, 1954). Two entirely unrelated antimalarials apparently antagonized the action of acetylcholine in the same way and changed the action of acetylcholine on the atria from the usual inhibitory action to a stimulant one. Experiments were therefore made to see if chloroquine, mepacrine and pyrimethamine, which all possessed a quinidine-like action on the isolated electrically-driven rabbit atria, showed similar antagonism.

METHODS

Isolated Perfused Rabbit Heart.—The heart was removed from a freshly killed rabbit and perfused with Locke solution at 36–37° C. by the Langendorff method. A record was taken of the amplitude of the ventricular contractions. Normal Locke solution, containing NaCl 9.0 g., KCl 0.42 g., $CaCl_2$ 0.2 g., dextrose 1.0 g., $NaHCO_3$ 0.5 g., distilled water to 1,000 ml., was used for all experiments except those on atropine, when a slightly modified solution described by McEwen (1956) was used.

Isolated Rabbit Atria.—The atria were carefully dissected from ventricular muscle and as much fat and connective tissue as possible removed, particular care being taken to avoid damage to the atria in the pacemaker region. They were suspended in Locke solution containing twice the normal quantity of dextrose at 29° C., in a bath of 35 ml. capacity. Contractions were recorded by a straw lever, the natural frequency of which was much greater than the highest rate of contraction.

Results

Isolated Perfused Rabbit Heart.—The effect of adding atropine sulphate (10^{-6} g./ml.) to the Locke solution perfusing an isolated rabbit heart is shown in Fig. 1. The ventricular contractions were diminished in amplitude to a small fraction of their initial size in the course of 2–3 min.

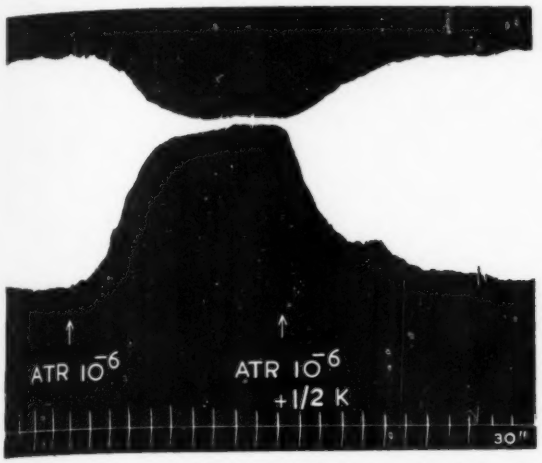

FIG. 1.—Contractions of isolated rabbit heart perfused by the Langendorff method. Record shows the effect of perfusing the heart with atropine 10^{-6} g./ml. in normal McEwen's solution. The amplitude was reduced to negligible proportions, but this depression was fully reversed on changing the perfusion fluid to McEwen's solution containing half the normal K^+.

When the perfusion fluid was changed to one containing half the K^+ concentration, but otherwise the same, this diminution was almost completely abolished.

Similar results were obtained with quinidine as shown in Fig. 2. Quinidine sulphate 3×10^{-5} g./ml. was added to the Locke solution and perfused through the heart. The amplitude was diminished as shown. The injection of 50 μg. and 100 μg. acetylcholine (ACh) into the cannula caused a very slight increase in the amplitude, and then a change to a perfusion fluid with one quarter the normal K^+ concentration produced complete recovery.

In another experiment, mepacrine hydrochloride in a concentration of 10^{-5} g./ml. reduced the amplitude and rate; this effect was abolished, though only partially, on changing to a perfusion fluid containing one quarter the concentration of K^+.

The action of chloroquine, proguanil and pyrimethamine on the isolated perfused rabbit heart was not investigated.

Isolated Rabbit Atria.—The action of quinidine on the isolated atria is clearly illustrated in Fig. 3. 0.5 mg. quinidine produced a gradual reduction in amplitude and rate. After 24 min. the beat was almost arrested, but on changing the fluid in the bath to quinidine in Locke solution containing a quarter the normal K^+ concentration, the amplitude and rate rapidly improved and became greater than at the beginning of the experiment. When conditions were steady, the bath fluid was changed back to Locke solution containing the normal K^+ concentration and the beat was arrested 12 min. later. The beat started again when the K^+ in the external medium was lowered to a quarter of the normal amount.

Pyrimethamine behaved in a similar way to quinidine. Fig. 4 shows the action of 1 mg. pyrimethamine in arresting the contractions of the atria in 15 min. and the beat restarting on changing the bath fluid to pyrimethamine in Locke solution containing a quarter the normal K^+ concentration. In section (c) after 10 min. exposure to the low K^+ concentration, when the amplitude was still small, 0.2 mg. acetylcholine caused a large increase in amplitude.

The depressant effect of chloroquine and mepacrine was also partly abolished by reducing the K^+ concentration of the solution. This is illustrated for chloroquine in Fig. 5. The longer these substances were left in the bath, the more difficult it was to reverse their effect, and when once the beat was arrested a good recovery was never

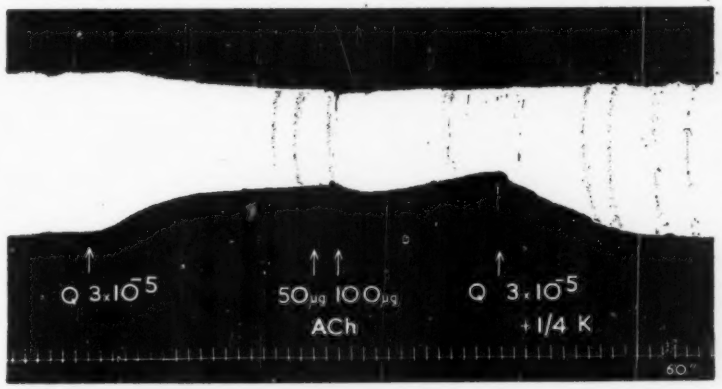

FIG. 2.—Record as in Fig. 1. The first part of the record shows the depressant effect of quinidine 3×10^{-5} g./ml. in normal Locke solution. In the presence of the quinidine, 100 μg. acetylcholine is seen to have first an inhibitory effect followed by a slight stimulant effect. On changing the perfusion fluid to one containing one quarter of the normal K^+, the amplitude returned to its original level.

FIG. 3.—Spontaneous contractions of isolated rabbit atria in a 35 ml. bath at 29° C. (a) At Q, 0·5 mg. quinidine in normal Locke solution. (b) 24 min. later, at first arrow, bath fluid changed to Locke solution containing one quarter of the normal K^+ but the same concentration of quinidine. At second arrow, bath fluid changed again to normal Locke solution + quinidine. (c) 12 min. later, the beat was arrested, but started again when the K^+ concentration in the external medium was lowered to one quarter.

obtained merely by lowering the K^+ concentration.

In three trials on two atria, the depression produced by proguanil (10^{-4} g./ml.) could not be reversed by lowering the K^+ concentration, either before or after the beat had stopped. In this respect, proguanil differed from the other antimalarial compounds tested.

Antagonism of Antimalarials by Acetylcholine.—From observations on 18 atria, it was found that chloroquine, mepacrine, and pyrimethamine, when added to the Locke solution in which the atria were beating, reduced the amplitude and rate of beat. In sufficient dose, each antimalarial compound arrested the beat within 5 to 60 min. Doses of 0.5–1.0 mg. of mepacrine or pyrimethamine in a 35 ml. bath generally arrested the beat after about 30 min. Chloroquine was less active, and a dose of about 4 mg. was required to arrest the beat in a similar time. There was usually a gradual diminution in amplitude and rate of beat during exposure to chloroquine, mepacrine and pyrimethamine. In this behaviour, they differed from proguanil, which usually arrested the beat suddenly.

After arrest by proguanil or quinidine, addition of acetylcholine always restarted the atrial contractions. After arrest by chloroquine, mepacrine,

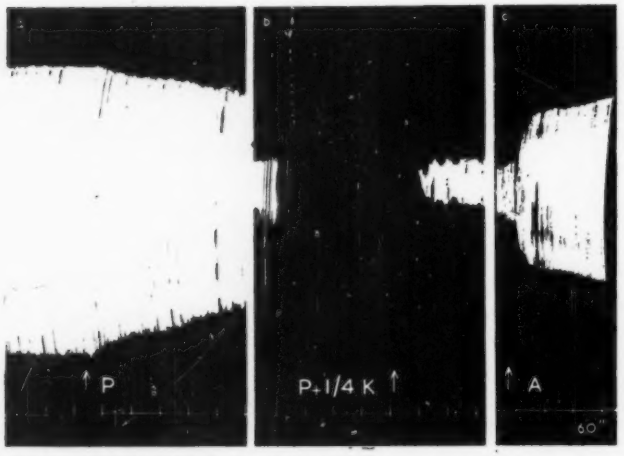

FIG. 4.—Record as in Fig. 3. (a) At P, 1 mg. pyrimethamine in normal Locke solution. (b) 15 min. later, beat arrested, but restarted on changing bath fluid to Locke solution containing one quarter of the normal K^+, but with the same concentration of pyrimethamine. (c) 10 min. later at A, 0·2 mg. acetylcholine. Note large increase in amplitude.

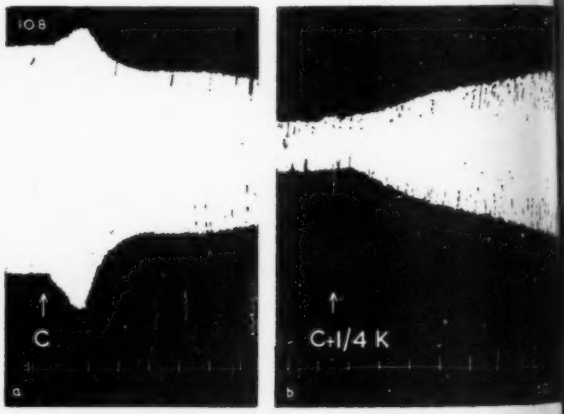

FIG. 5.—Record as in Fig. 3. The numerals at the top of the record are the number of beats/min. (a) At C, 1 mg. chloroquine in normal Locke solution. (b) 20 min. later, amplitude and rate greatly depressed. Bath fluid changed to Locke solution containing one quarter of the normal K^+, but the same concentration of chloroquine.

or pyrimethamine, however, addition of acetylcholine restored the beat in only one out of eight attempts with pyrimethamine, two out of six attempts with mepacrine and none out of three attempts with chloroquine. If the drug was first removed from the bath, acetylcholine always restarted the contractions. That the acetylcholine was responsible and not the removal of the antimalarial compound was shown by the fact that, when the acetylcholine was removed by washing out the bath, the beat was sometimes arrested again.

Before the contractions were arrested, a dose of acetylcholine which had initially caused a considerable reduction in amplitude and rate was usually without effect 10 min. after the addition of chloroquine, mepacrine, or pyrimethamine in a concentration of 10^{-5} to 3×10^{-5} g./ml. After a longer period, acetylcholine was usually observed to increase the size of the atrial contractions as shown in Fig. 6. This abolition of the inhibitory action of acetylcholine followed by conversion to a stimulant action under the influence of chloroquine, mepacrine, and pyrimethamine was identical with the behaviour of proguanil and quinidine.

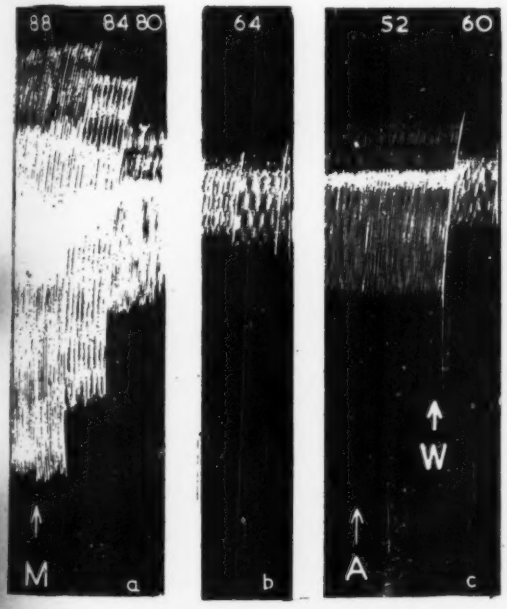

FIG. 6.—Record as in Fig. 3. The numerals at the top of the record are the number of beats/min. (a) At M, 0·7 mg. mepacrine. Amplitude and rate reduced. Rates after 5 and 10 min. (b) Half an hour later. Amplitude and rate further reduced but beat not arrested. (c) 0·6 mg. acetylcholine was added to the bath over 15 min. The last 0·1 mg. was added at A. Amplitude increased and rate hardly affected by this amount of acetylcholine. After change of bath fluid at W, amplitude and rate returned to those shown in (b).

DISCUSSION

When atropine, quinidine or mepacrine were added to the fluid perfusing the isolated rabbit heart, the amplitude was greatly diminished, and quinidine and mepacrine in addition slowed the rate. When the concentration of K^+ in the fluid was reduced these effects were reversed. Similar observations were made on isolated atria after adding either quinidine, pyrimethamine, chloroquine or mepacrine to the bath. The amplitude of beat was reduced, but the effect was reversed on changing to a fluid with a lower concentration of K^+.

During contraction potassium passes out of the cell through the depolarized and permeable cell membrane by diffusion. The foregoing observations suggest that, when perfused through the heart or when acting on the atria, quinidine renders the cell membrane less permeable to potassium ions. In consequence the efflux of K^+ is diminished and repolarization therefore becomes less and less complete. For when the external concentration of K^+ is lowered to one-half or one-quarter, the gradient for the efflux of K^+ is increased, and this compensates for the effect of quinidine. Such an explanation of the action of quinidine serves also to explain the action of acetylcholine in restarting the contractions of atria arrested by quinidine. Burgen and Terroux (1953) and Hoffman and Suckling (1953) showed that the action potential of the isolated atria was characterized by a slow rate of repolarization, and that this rate was accelerated by acetylcholine. Burgen and Terroux suggested that acetylcholine facilitated the efflux of K^+, presumably by making the membrane more permeable to K^+, and such an increased permeability has now been observed by Harris and Hutter (1956) using radioactive K^+. The stimulant action of acetylcholine in restarting the contractions when they have been arrested by quinidine is thus explained by its action in increasing the permeability to K^+.

Pyrimethamine, chloroquine and mepacrine exerted a similar depressant effect on the atrial contractions which was also reversed by changing to a fluid low in K^+. The depression caused by proguanil was not reversed in this way; but this depression, even when it proceeded as far as arrest, was always reversed by acetylcholine. Atria arrested by proguanil were shown by Burn and Vane (1949) to respond to electrical stimulation. In the present experiments it was found that, after arrest by chloroquine, mepacrine or pyrimethamine, the atria did not respond to electrical

stimulation, but after arrest by quinidine they usually did respond. Thus the evidence indicates that while the action of proguanil and quinidine may differ somewhat from that of the other substances, a diminution in the permeability of the membrane to potassium is largely responsible for causing arrest of the atria.

The particular interest of these findings lies in their application to the use of quinidine in fibrillation. Both in atrial and in ventricular fibrillation there is an increased efflux of K^+. In ventricular fibrillation of the isolated rabbit heart this has been directly observed (Armitage, Burn, and Gunning, 1957). In atrial fibrillation it is suggested by the fact that this fibrillation is arrested when the plasma K^+ concentration is gradually raised (Burn, Gunning, and Walker, 1956). This appears to be the first time that atrial fibrillation has been arrested by raising the external K^+ concentration. It is interesting to note, however, that the use of injections of potassium salts (0.1% KCl solution) in arresting ventricular fibrillation was described by Hering as long ago as 1903. The action of quinidine in arresting fibrillation may therefore be due to its effect in diminishng the permeability of the cell membrane to K^+, and thus lessening the efflux of K^+.

I wish to thank Professor J. H. Burn for his supervision and advice throughout this work, and particularly for his help in the presentation of the paper. I am also indebted to the Wellcome Trustees for a personal grant.

References

Armitage, A. K., Burn, J. H., and Gunning, A. J. (1957). *Circulation Research*, **5**, 98.

Briscoe, S., and Burn, J. H. (1954). *Brit. J. Pharmacol.*, **9**, 42.

Burgen, A. S. V., and Terroux, K. G. (1953). *J. Physiol.*, **120**, 449.

Burn, J. H., Gunning, A. J., and Walker, J. M. (1956). *Circulation Research*, **4**, 288.

—— and Vane, J. R. (1949). *J. Physiol.*, **108**, 104.

Harris, E. J., and Hutter, O. F. (1956). Ibid., **133**, 58 P.

Hering, H. E. (1903). *Zbl. Physiol.*, **17**, 1.

Hoffman, B. F., and Suckling, E. E. (1953). *Amer. J. Physiol.*, **173**, 312.

McEwen, L. (1956). *J. Physiol.*, **131**, 678.

Brit. J. Pharmacol. (1957), **12**, 79.

THE ACTION OF MORPHINE ON THE SUPERIOR CERVICAL GANGLION AND ON THE NICTITATING MEMBRANE OF THE CAT

BY

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(RECEIVED OCTOBER 5, 1956)

The intravenous injection of 0.05 to 2.5 mg. of morphine reduced the response of the nictitating membrane in the cat to pre- and post-ganglionic stimulation. This inhibitory action of morphine was due neither to inhibition of ganglionic transmission nor to a depressant action on the smooth muscle of the nictitating membrane. It is suggested that morphine inhibits the release of the sympathetic transmitter from the postganglionic nerve endings.

Small amounts of morphine (5 to 20 μg.) injected intravenously reduced or abolished the contraction of the nictitating membrane due to the injection of histamine, pilocarpine and 5-HT into the arterial blood supply of the superior cervical ganglion. This inhibitory action of morphine was due to an action on the ganglion cells, since such small amounts of morphine did not reduce the response of the nictitating membrane to postganglionic stimulation. Similar amounts of morphine did not abolish the stimulation of the ganglion by nicotine, tetramethylammonium and potassium chloride.

The results provide further evidence for the view that histamine, pilocarpine, and 5-hydroxytryptamine have no "nicotine-like" properties but act on receptors of the ganglion cells different from the acetylcholine receptors.

Rocha e Silva, Valle and Picarelli (1953) were the first to observe that the stimulation of the isolated guinea-pig ileum by 5-hydroxytryptamine (5-HT) was due to an action of 5-HT on some nervous mechanism and not to a direct action on the longitudinal muscle. Further evidence for this view was provided by observations by Robertson (1953) and by Gaddum and Hameed (1954). The latter concluded that 5-HT acted on specific tryptamine receptors of the intestinal ganglion cells. Kosterlitz and Robinson (1955) found that small amounts of morphine abolished the response of the isolated guinea-pig ileum to 5-HT.

As it had been observed that 5-HT stimulated the superior cervical ganglion of the cat (Robertson, 1953; Trendelenburg, 1956a), it was of interest to investigate the effect of morphine on this nervous action of 5-HT. Histamine and pilocarpine have ganglionic actions very similar to those of 5-HT (Trendelenburg, 1954, 1955, 1956b); they were therefore included in this study.

METHODS

Cats of 2 to 5 kg. of both sexes were used. After inducing anaesthesia with ether, 80 mg./kg. chloralose was injected intravenously. Intra-arterial injections "to the ganglion" were made through the central end of the lingual artery during occlusion of the external carotid artery. The injected substance was thus diverted to the superior cervical ganglion. Injections "to the nictitating membrane" were made similarly, but without occlusion of the external carotid artery; the injected substance then reached the nictitating membrane (Trendelenburg, 1954).

For postganglionic stimulation, the sympathetic trunk was exposed by removing the larynx, part of the oesophagus, and the *M. longus colli*. A unipolar electrode was hooked round the postganglionic sympathetic fibres at a distance of 2.4 mm. from the superior cervical ganglion. For preganglionic stimulation the cervical sympathetic chain was exposed, cut, and its peripheral end was placed on shielded electrodes. The nerve was then covered with warm liquid paraffin. In all experiments, the connexions of the superior cervical ganglion to the centres were divided by cutting the preganglionic fibres.

The movements of the nictitating membrane were recorded by attaching it to an isotonic lever fitted with a frontal writing point. Intravenous injections were made through the femoral vein.

For intra-arterial injections, the solutions were neutralized. The following substances were used:

morphine sulphate, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, pilocarpine nitrate, nicotine hydrogen tartrate, and tetramethylammonium bromide ; all weights refer to the salts.

Results

Response of the Nictitating Membrane to Preganglionic Stimulation.—Morphine reduced the response of the nictitating membrane to submaximal preganglionic stimulation of the cervical sympathetic nerve. This was observed in eight preparations when intravenous injections of 20 μg., 100 μg., and 500 μg. morphine were given at 60 min. intervals. After 20 μg. the diminution was 18%, after 100 μg. it was 30% and after 500 μg. it was 52%. In these experiments preganglionic stimulation was applied every 30 sec. for 5 sec. The rate of stimulation was 2/sec. or 15/sec. The duration of the inhibitory action of morphine could not be determined accurately, as the response of the nictitating membrane to submaximal preganglionic stimulation usually failed to remain constant for periods of more than 10 to 15 min. In a few experiments, however, full recovery of the response was observed about 20 min. after the intravenous injection of 20 μg. morphine.

Response of the Nictitating Membrane to Postganglionic Stimulation.—Hebb and Konzett (1949) showed that morphine did not block transmission through the perfused superior cervical ganglion of the cat, but some ganglionic effects, such as those of histamine, are not readily seen in the perfused preparation. Experiments were carried out in which electrical stimulation was applied alternately to the pre- and post-ganglionic fibres of the superior cervical ganglion. Fig. 1 shows that the response of the nictitating membrane to both types of stimulation was reduced to the same extent after the intravenous injection of 100 μg. morphine, so that the effect was not due to ganglion block. When electrical stimulation was applied to the peripheral end of the postganglionic fibres after cutting them, intravenous injections of morphine caused a reduction of the response of the nictitating membrane. The presence of the ganglion cells was thus not essential for this effect of morphine.

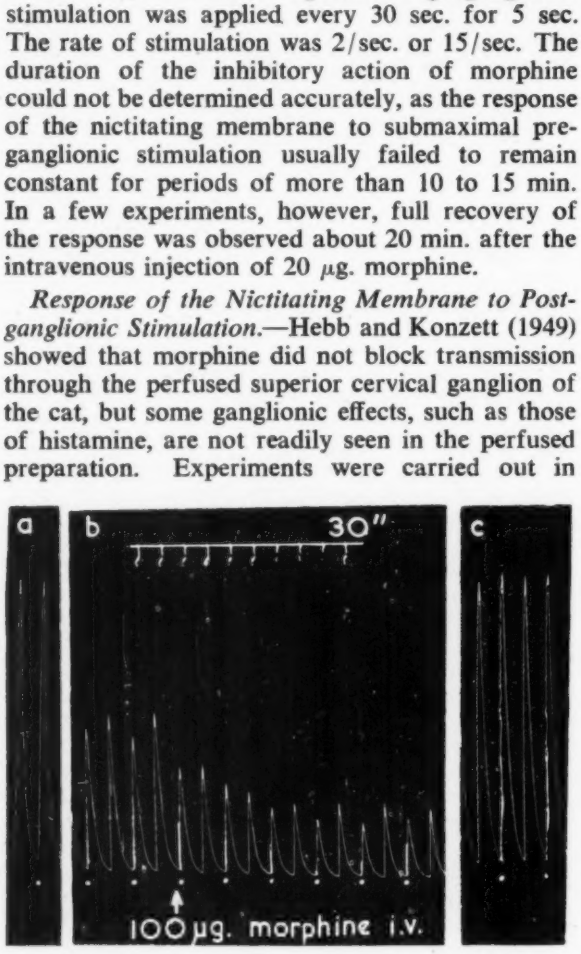

FIG. 1.—Cat, chloralose anaesthesia, 4 kg. Record of nictitating membrane. Electrical stimulation for 5 sec. twice per min., alternately applied to pre- and post-ganglionic fibres (post-ganglionic stimulation marked with dots). Strength of stimulation supramaximal in (a) and (c), submaximal in (b). Rate of stimulation 2/sec. Intravenous injection of 100 μg. morphine in (b). Record (c) was taken 2 min. after (b).

Fig. 1 also shows that the response of the nictitating membrane to supramaximal stimulation of either the pre- or the post-ganglionic fibres was scarcely affected by the injection of 100 μg. morphine (compare Fig. 1a and c).

Influence of the Height of Contraction of the Nictitating Membrane.—Morphine depressed the response of the nictitating membrane to pre- or post-ganglionic stimulation to a greater extent when the stimulation rate was 2/sec. than when it was 15/sec. Supramaximal stimulation applied for 5 sec. at a rate of 2/sec. caused much smaller contractions of the nictitating membrane than supramaximal stimulation applied for the same period at the faster rate of 15/sec. In some experiments supramaximal stimulation (2/sec.) was applied alternately with submaximal stimulation (15/sec.) to the pre- or post-ganglionic fibres. The strength of submaximal stimulation was chosen so as to produce contractions of the nictitating membrane of similar height to those produced by supramaximal stimulation at 2/sec. Intravenous injections of 0.2 to 2.5 mg. morphine then reduced the response of the nictitating membrane to both types of stimulation to the same degree. Thus the response of the nictitating membrane to either pre- or post-ganglionic stimulation was the more reduced, the smaller the initial response of the nictitating membrane ; the rate of stimulation influenced the magnitude of the depressing effect of morphine only in so far as it determined the height of the contraction of the nictitating membrane.

Response of the Nictitating Membrane to Adrenaline and Noradrenaline.—Fig. 2 shows that the intravenous injection of 0.5 to 2.5 mg. morphine reduced the response of the nictitating membrane to submaximal preganglionic stimulation (Fig. 2c) to a much greater extent than the response to supramaximal stimulation (Fig. 2a

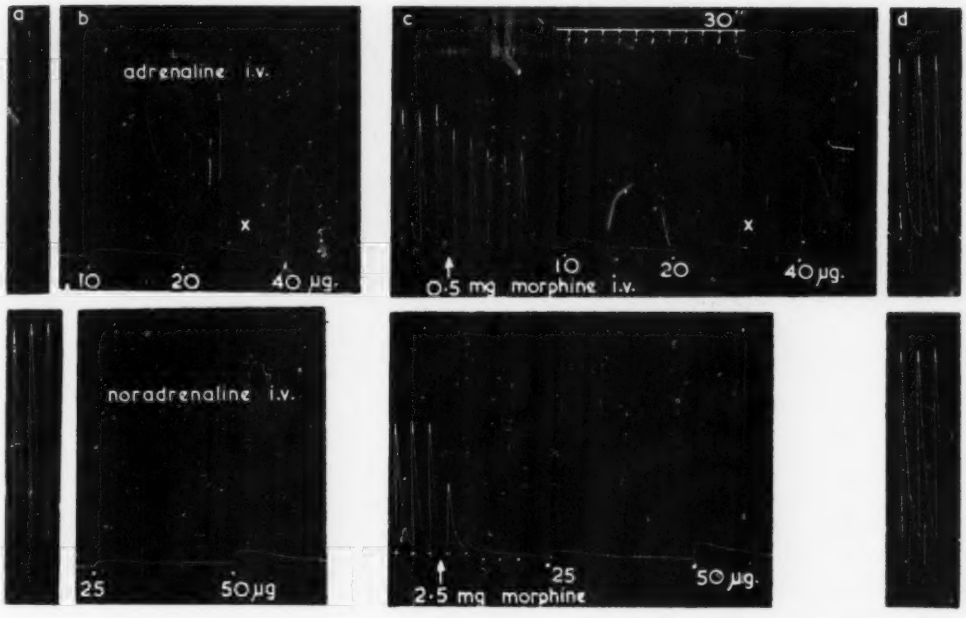

FIG. 2.—Cat, chloralose anaesthesia, 2 kg. Record of nictitating membrane. Preganglionic stimulation applied for periods of 5 sec. twice per min. Rate: 2/sec. Strength of stimulation: supramaximal in (a) and (d), submaximal in (c). (Upper trace.) Intravenous injections of 10, 20, and 40 µg. adrenaline (b) before and (c) after the intravenous injections of 0.5 mg. morphine. Lower trace: intravenous injections of 25 and 50 µg. noradrenaline (b) before and (c) after intravenous injection of 2.5 mg. morphine. Between the injections of 20 and 40 µg. adrenaline the drum was stopped for 5 min. (X). Record (d) was taken 2 min. after record (c).

and d). Although the contractions of the nictitating membrane caused by intravenous injections of adrenaline and noradrenaline (Fig. 2b) were smaller than those caused by submaximal preganglionic stimulation, the effect of adrenaline and noradrenaline was not reduced by morphine (Fig. 2c). After intravenous injections of as much as 10 mg. morphine, the response of the nictitating membrane to intravenous injections of adrenaline and noradrenaline was slightly increased.

Stimulation of the Superior Cervical Ganglion by Nicotine-like Substances and by Potassium Chloride.—Injections of 2.5 to 10 µg. nicotine or tetramethylammonium or of 1 to 2 mg. potassium chloride into the blood supply of the superior cervical ganglion caused submaximal contractions of the nictitating membrane due to stimulation of the ganglion cells. Intravenous injections of 100 µg. and 500 µg. morphine depressed the response of the nictitating membrane to these injections to about the same extent (20% and 53% respectively) as the response of the nictitating membrane to submaximal pre- or post-ganglionic stimulation.

The response of the nictitating membrane to the intra-arterial injection of larger amounts of nicotine, tetramethylammonium or potassium chloride,

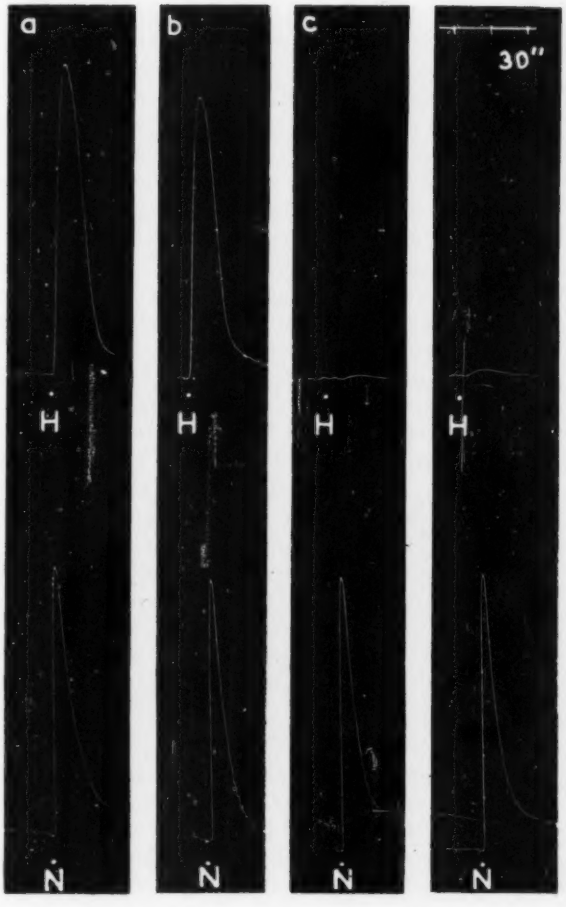

FIG. 3.—Cat, chloralose anaesthesia, 5 kg. Nictitating membranes. Intra-arterial injections to the left ganglion of 5 µg. histamine (H) and to the right ganglion of 40 µg. nicotine (N). Intravenous injection of 1 mg. morphine between (b) and (c). Time intervals of 20 min. between records.

FIG. 4.—Cat, chloralose anaesthesia, 4.5 kg. Nictitating membrane. Intra-arterial injections to the ganglion of 20 µg. histamine at arrow. In (c) submaximal postganglionic stimulation applied at a rate of 2/sec. for periods of 5 sec. once per min. Intravenous injection of 30 µg. morphine in (c). Intervals of 20 min. between records.

which caused a maximal contraction of the nictitating membrane, was reduced only slightly or not at all after intravenous administration of 0.2 to 1 mg. morphine. Such an experiment is shown in Fig. 3 (lower trace).

Stimulation of the Superior Cervical Ganglion by Histamine, Pilocarpine and 5-HT.—The following observations concern a different action of morphine on the ganglion. Fig. 3 illustrates the fact that morphine abolished the response of the nictitating membrane to intra-arterial injections of 5 µg. histamine, while it did not affect the response to nicotine, although the contractions of the nictitating membrane caused by both histamine and nicotine were initially of similar height.

The response of the nictitating membrane to intra-arterial injections to the ganglion of pilocarpine and 5-HT was also abolished after intravenous injections of morphine. The amounts of morphine required were small; 5 µg. morphine usually reduced the action of these substances, and 20 to 30 µg. morphine abolished it. Fig. 4 shows an experiment in which the intravenous injection of 30 µg. morphine caused a very small reduction of the response of the nictitating membrane to submaximal postganglionic stimulation (Fig. 4c); but the response of the nictitating membrane to the intra-arterial injection of 20 µg. histamine was completely abolished (Fig. 4d). Partial recovery of the response to histamine was observed during the following 2 hr. (Fig. 4e to h). The reduction of the action of histamine, pilocarpine and 5-HT on the superior cervical ganglion was thus of much longer duration than the slight reduction of the response of the nictitating membrane to nerve stimulation caused by these small amounts of morphine.

The long duration of the action of morphine is also shown in Fig. 5. The intravenous injection of 20 µg. morphine reduced the response of the nictitating membrane to very weak submaximal preganglionic stimulation and abolished the response to intra-arterial injections of 20 µg. pilocarpine. Recovery of the response to pilocarpine was not complete until 3 to 4 hr. after the injection of morphine. The figure also shows that mor-

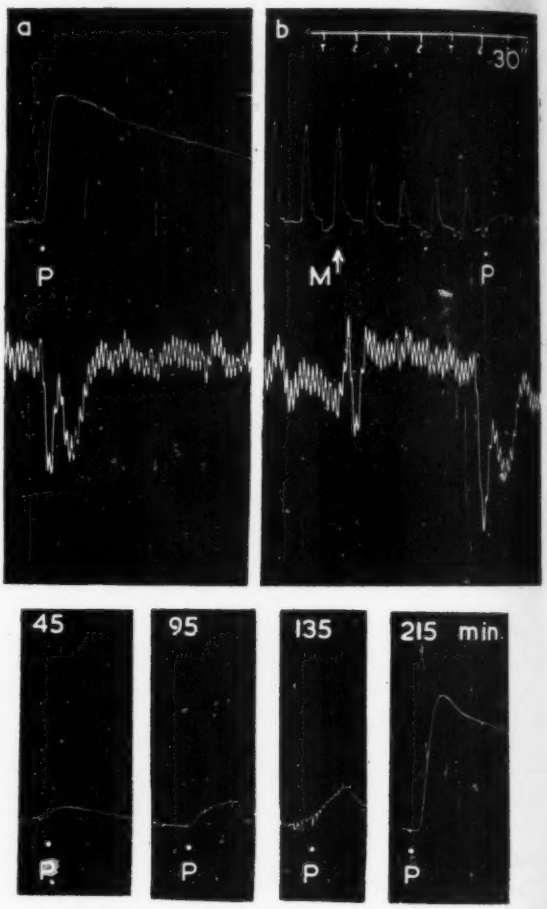

FIG. 5.—Cat, chloralose anaesthesia, 4.1 kg. Nictitating membrane and arterial blood pressure. Intra-arterial injections to the ganglion of 20 µg. pilocarpine (P). Submaximal preganglionic stimulation at a rate of 2/sec. for periods of 5 sec. twice per min. in (b). Intravenous injection of 20 µg. morphine at (M) in (b); the lower 4 records were obtained 45, 95, 135, and 215 min. after the injection of morphine.

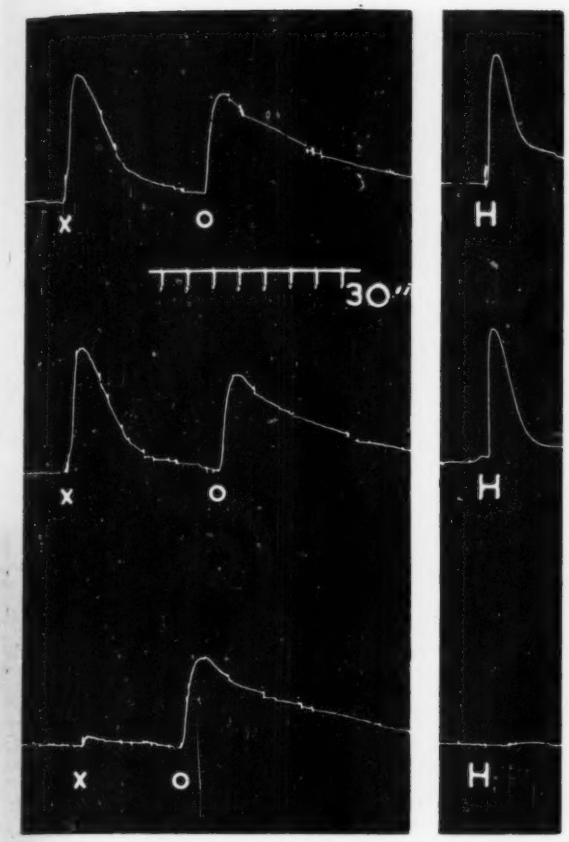

FIG. 6.—Cat, chloralose anaesthesia, 2.1 kg. Nictitating membrane. Intra-arterial injection of 10 μg. 5-HT to the ganglion (X) and to the nictitating membrane (○), intra-arterial injection to the ganglion of 5 μg. histamine (H). The upper and middle traces are before, and the lower trace is after, the intravenous injection of 50 μg. morphine. Time interval of 30 min. between traces.

phine failed to reduce the fall of blood pressure caused by pilocarpine. Likewise the depressor action of histamine was not antagonized.

In contrast to histamine and pilocarpine, 5-HT has a direct action on the smooth muscle of the nicititating membrane (Trendelenburg, 1956a). Fig. 6 shows that morphine abolished the response of the nictitating membrane caused by intra-arterial injections to the ganglion of 10 μg. 5-HT and 5 μg. histamine; but the direct action of 5-HT (injected intra-arterially at O) on the smooth muscle of the nictitating membrane remained unchanged.

When morphine was injected intra-arterially into the blood supply of the ganglion, it was found to be active in much smaller amounts. Fig. 7 shows that the intra-arterial injection of 0.5 μg. morphine abolished the action of histamine on the ganglion; when, however, the same amount of morphine was injected intravenously, it failed to alter the action of histamine (Fig. 7f). Similar observations were made when 0.5 μg. morphine was injected first intravenously and then intra-arterially.

Potentiating Effects of Histamine, Pilocarpine, and 5-HT.—These three substances potentiate the response of the nictitating membrane to submaximal preganglionic stimulation, by facilitating transmission through the superior cervical ganglion (Trendelenburg, 1955, 1956a, b, and unpublished observations). Fig. 8 shows that morphine antagonized this potentiating action. 10 μg. each of pilocarpine (P), 5-HT (HT) and of histamine (H) were injected intra-arterially during intermittent submaximal stimulation of the preganglionic fibres. The intra-arterial injections were made at 20 min. intervals; they failed to stimulate the superior cervical ganglion, but regularly caused pronounced potentiation of the response of the nictitating membrane (Fig. 8b to f). After the intravenous injection of 50 μg. morphine (between f and g), the potentiating effects of the three substances were much reduced (Fig. 8g to i), while the response of the nictitating membrane to supramaximal preganglionic stimulation was scarcely altered (compare Fig. 8a and k). In order to abolish the potentiating effect of histamine, pilocarpine and 5-HT on ganglionic transmission, larger amounts of morphine (100 to 200 μg.) had to be injected intravenously than those required for abolition of the ganglion-stimulating action of these three substances.

DISCUSSION

The present results have revealed two actions of morphine, one on some part of the postganglionic axon peripheral to the electrodes used for post-ganglionic stimulation, and the other on the superior cervical ganglion.

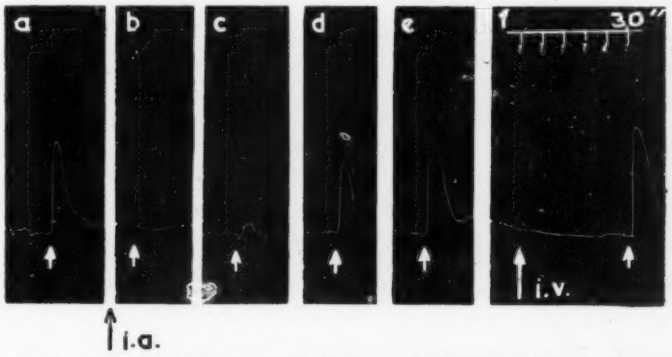

FIG. 7.—Cat, chloralose anaesthesia, 2.1 kg. Nictitating membrane. Intra-arterial injections to the ganglion of 10 μg. histamine at arrow. Injections of 0.5 μg. morphine, intra-arterially to the ganglion between (a) and (b), intravenously in (f). Time intervals of 20 min. between records.

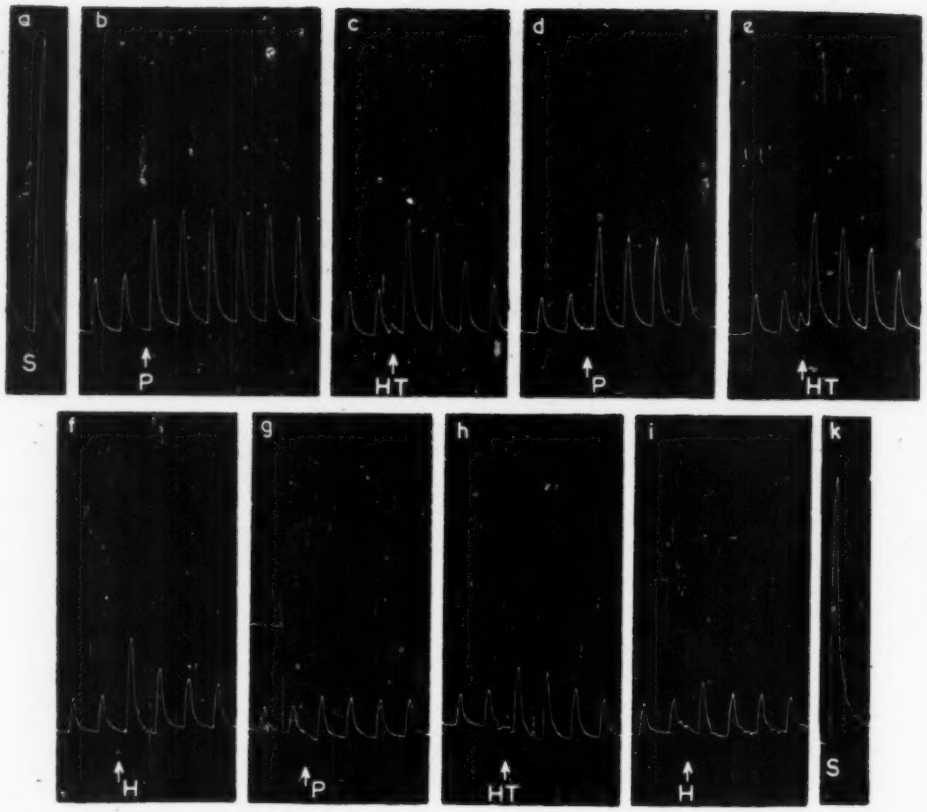

FIG. 8.—Cat, chloralose anaesthesia, 4 kg. Nictitating membrane. Preganglionic stimulation applied at a rate of 15/sec. for periods of 5 sec. twice per min. Supramaximal strength in (a) and (k), submaximal in (b) to (i). Intra-arterial injections to the ganglion of 10 μg. pilocarpine (P), 10 μg. 5-HT (HT) and 10 μg. histamine (H). Intravenous injection of 50 μg. morphine between (f) and (g). Time intervals of 20 min. between records (b) to (i).

Larger amounts of morphine (50 μg. to 2.5 mg.) depressed the response of the nictitating membrane to pre- and post-ganglionic stimulation to the same degree. Morphine thus did not block the transmission of nerve impulses through the superior cervical ganglion. Nor did it antagonize the action of nicotine-like substances and of potassium chloride on the superior cervical ganglion. The response of the nictitating membrane to intra-arterial injections of these ganglion-stimulating substances and that to preganglionic stimulation was equally reduced by morphine, provided the strength of electrical stimulation was chosen so as to produce contractions of the nictitating membrane of a similar height.

The inhibition by morphine of the response of the nictitating membrane to pre- and post-ganglionic stimulation was not due to an action of morphine on the smooth muscle of the nictitating membrane, since the direct action of adrenaline, noradrenaline and 5-HT on the nictitating membrane was not reduced.

As morphine was found to have neither a ganglion-blocking action nor a direct depressant effect on the nictitating membrane, it seemed it might act by reducing the amount of sympathetic transmitter liberated from the postganglionic nerve endings on stimulation of the pre- or post-ganglionic fibres. Such an assumption is supported by the observation (Paton, personal communication) that morphine reduces the liberation of acetylcholine from the isolated intestine on direct electrical stimulation of the wall of the intestine, which is believed to stimulate the post-ganglionic fibres embedded in the tissue.

The inhibitory action of morphine was found to be related to the height of the contractions of the nictitating membrane caused by pre- or post-ganglionic stimulation or by intra-arterial injections into the blood supply of the ganglion of nicotine-like substances or of potassium chloride. As the periods of stimulation were kept constant (5 sec.), it is likely that the height of the contraction of the effector organ was related to the amount

of sympathetic transmitter liberated per unit time. This amount was increased either by stimulating more nerve fibres with stronger currents or by increasing the frequency of stimulation from 2/sec. to 15/sec. The larger the initial contraction of the nictitating membrane the less pronounced was the depressing effect of morphine.

It has recently been pointed out that histamine, pilocarpine and 5-HT stimulate the superior cervical ganglion of the cat by combining with receptors, which differ from the acetylcholine receptors of the ganglion cells. These three substances therefore cannot be considered as having "nicotine-like" properties (Trendelenburg, 1956b). The present results provide further evidence for the view that histamine, pilocarpine and 5-HT differ from both the "nicotine-like" ganglion-stimulating substances and from potassium chloride. Small amounts of morphine, which were found to interfere neither with the liberation of sympathetic transmitter from the postganglionic nerve endings nor with the ganglion-stimulating action of nicotine-like substances and of potassium chloride, caused long-lasting depression of the response of the superior cervical ganglion to histamine, pilocarpine and 5-HT. The direct action of 5-HT on the smooth muscle of the nictitating membrane, on the other hand, was not affected by morphine. This observation agrees with recent findings reported to the British Pharmacological Society in July, 1956, by Picarelli that the guinea-pig ileum contains two types of specific tryptamine-receptors, one of which has been found to be blocked by morphine.

Previous results had shown that cocaine had an action rather similar to that of morphine, in so far as cocaine antagonized the ganglionic actions of histamine, pilocarpine and 5-HT without interfering with ganglionic transmission or the ganglion-stimulating action of nicotine-like substances and of potassium chloride (Trendelenburg, 1954, 1956a). The minimal effective dose of intravenous injections of cocaine was found to be 0.1 to 0.5 mg., while morphine was effective in doses of 5 to 20 μg. Cocaine was thus about 20 times less active than morphine when administered by the same route. Picarelli (1956) has found that cocaine antagonized the response of the isolated guinea-pig ileum to 5-HT, and that this was due to an interference with the action of 5-HT on the morphine-sensitive tryptamine receptors. Cocaine failed to antagonize the response of the ileum to 5-HT when the morphine-sensitive tryptamine receptors had been blocked by the addition of morphine to the bath. These observations provided further evidence for the view that morphine and cocaine resemble each other in their antagonistic action towards 5-HT.

References

Gaddum, J. H., and Hameed, K. A. (1954). *Brit. J. Pharmacol.*, **9**, 240.

Hebb, C. O., and Konzett, H. (1949). *Quart. J. exp. Physiol.*, **35**, 213.

Kosterlitz, H. W., and Robinson, J. (1955). *J. Physiol.*, **128**, 18P.

Robertson, P. A. (1953). Ibid., **121**, 54P.

Rocha e Silva, M., Valle, J. R., and Picarelli, Z. P. (1953). *Brit. J. Pharmacol.*, **8**, 378.

Trendelenburg, U. (1954). Ibid., **9**, 481.

—— (1955). *J. Physiol.*, **129**, 337.

—— (1956a). *Brit. J. Pharmacol.*, **11**, 74.

—— (1956b). *J. Physiol.*, **132**, 529.

Brit. J. Pharmacol. (1957), **12**, 86.

THE TRANSMITTER RELEASED BY STIMULATION OF THE BRONCHIAL SYMPATHETIC NERVES OF CATS

BY

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A study has been made of the transmitter released in a cat heart-lung preparation when the sympathetic chains were stimulated. The nervi accelerantes were always sectioned before stimulation. The transmitter appeared first in the pulmonary venous blood. In its actions on the heart-lung preparation, it resembled isoprenaline and not adrenaline. Chromatographic studies using three different solvents showed that 80 to 100% of this transmitter consisted of a catechol amine which had R_F values which were identical with those of isoprenaline. Pharmacological studies failed to distinguish between the actions of this amine and those of isoprenaline, but clearly differentiated between those of the pulmonary amine, adrenaline, and noradrenaline.

The discovery of trace amounts of a third sympathomimetic amine in saline extracts of the adrenal glands of cats, monkeys, and man has been reported by Lockett (1954). This amine could not be differentiated from isoprenaline (*N*-isopropylnoradrenaline) by its colour reactions, by its chromatographic behaviour, or by its pharmacological activity; it could, however, readily be distinguished from adrenaline and from noradrenaline both in R_F values and by pharmacological means.

The possible physiological significance of this trace amine required study. The chemical transmitter of the postganglionic sympathetic nerve fibres supplying bronchi was examined because isoprenaline (Konzett, 1940a) and the third amine of the adrenal gland (Lockett, 1954) had proved more active than adrenaline as dilators of previously constricted bronchioles. Cats were selected for this investigation because the third amine of the adrenals was known to occur in this species (Lockett, 1954).

METHODS

The experiments recorded involved 123 cats. Anaesthesia was induced with chloroform and ether (1:4 parts v/v) and was maintained with chloralose, 8 ml./kg. of a 1% w/v solution in 0.9% w/v NaCl in water, given by femoral venous cannula. The anticoagulant used was heparin (Liquemin, Roche), 500 units/kg. body weight. Donor blood, containing very little adrenaline and noradrenaline, was obtained as follows: anaesthetized cats were made spinal, the adrenals were excluded from the circulation, and the sympathetic chains were removed from just above the stellate ganglia to the brim of the pelvis. After an interval of 20 min. the cats were bled from a carotid arterial cannula.

Heart-lung preparations were made in adrenalectomized spinal cats by a technique essentially similar to that used by Knowlton and Starling (1912). Systemic outflow was recorded from a simple syphon placed between the peripheral resistance and the reservoirs. The circuit was arranged to allow the return of blood from the syphon recorded to either of two reservoirs. These reservoirs were small double surface condensers, each of 60 ml. maximum capacity. Blood from either reservoir was returned to the heart through a 4 in. Liebig condenser which adjoined the cannula in the superior vena cava. The reservoirs and the Liebig condenser were warmed with water which was circulated by a Stewart Turner aquarium pump from an electrically heated, thermostatically controlled water bath. Air pressure in the Starling type of peripheral resistance and brachiocephalic mean arterial pressure were recorded from mercury manometers in the usual way. Right atrial pressures and venous filling pressures were recorded from mercury manometers, the floats of which moved frontal writing levers; the latter magnified the movements of the mercury surfaces 8 to 13 times. Change in heart size was recorded by a cardiometer connected with a bellows recorder. Oxygen consumption was measured by means of a float recorder which formed part of an oxygen-filled closed circuit. A Starling pump supplied the preparation with oxygen drawn from this float, and returned the expired gas to it through a cannister filled with soda lime. Changes in bronchiolar resistance were inferred from records of tidal air made by Konzett's method (1940a).

Electrical Stimulation of the Sympathetic Chains.—Loose ligatures were first placed round the great

vessels ready for the establishment of the heart-lung preparation. Next, the nervi accelerantes were divided and the azygos vein and subclavian arteries were tied. Rapid incisions were then made through the sternum and intercostal spaces such that shielded Palmer electrodes could lie horizontally below the level of the great vessels. These electrodes were placed between the ganglia and round the sympathetic chains from just below the stellate ganglia to the neck of the 8th rib on each side. The wiring was so arranged that current should pass through each ganglion from one electrode to the next. Rectangular stimuli of 0.5 to 1 msec. duration were delivered at a rate of 12 to 16/sec. from a 5 to 10 v. source through all the electrodes on both sides simultaneously.

Electrical Stimulation of the Heart.—Hearts were driven at constant high rate by means of rectangular stimuli of 2 msec. duration delivered through small light shielded electrodes made of platinum wire mounted in perspex. These were screwed gently on to the edge of the right auricular appendage.

Concentrated Protein-free Extracts of Plasma.—These were prepared for chromatography by a method essentially similar to that described by Vogt (1952). Two modifications of this method were found advisable in adapting the method for use with plasma volumes which varied from 35 to 110 ml. First, the residues obtained after evaporation to dryness *in vacuo* were dissolved in known volumes of solvent, and the process was continued in each case with as large a measured volume of the solution as was practicable. Secondly, it was found necessary to repeat the extraction of the residues with ethanol saturated with sodium chloride.

Chromatography and Elution.—Concentrated acetone-alcohol extracts of plasma were quantitatively transferred to acid-washed Whatman No. 1 filter paper. The area of the paper to be traversed by plasma samples destined to be tested biologically was sprayed with aqueous ascorbic acid 50 mg./100 ml. In some experiments phenol containing 15% w/v 0.1N-HCl was used as solvent. In others the solvent was butanol saturated either with 0.5N-HCl (Crawford, 1951) or with 16.7% v/v aqueous acetic acid (Shepherd and West, 1953). Ascending chromatograms were made in an atmosphere of CO_2. Solvents were removed by washing with benzene, and the whole process, including drying, was carried out at room temperature.

Adrenaline, noradrenaline, and isoprenaline were used as reference compounds. 10 μg. of each amine was added to 20 ml. of control plasma, from which a protein-free concentrated extract of plasma was prepared. The alcohol-acetone extract of this plasma was added to a part of the paper washed with acid, but unsprayed with ascorbic. This practice was adopted because the R_F values given by these reference compounds when applied to the paper in plasma extract were usually slightly greater than the R_F values determined simultaneously for these amines applied to the paper in aqueous or alcohol-acetone solution (see Table I).

Chromatograms of one plasma sample for elution and of mixed reference compounds in plasma were made in parallel on one sheet of paper. The solvent front was allowed to advance beyond the line of application by not less than 16 cm. (phenol) or 22 cm. (butanol) to ensure good separation of the amines. The reference chromatogram was developed by spraying with 0.44 g. potassium ferricyanide dissolved in 100 ml. 0.2 M-phosphate buffer at *p*H 8.0 after drying. The methods used for elution and for the preparation of solutions for biological assay were described by Vogt (1952).

Drugs.—(−)-Adrenaline tartrate (Burroughs Wellcome), (−)-noradrenaline and (±)-isoprenaline (Sterling Winthrop) were obtained commercially.

Results

The Effect of Bilateral Stimulation of the Sympathetic Chains after Division of the Nervi Accelerantes in the Cat Heart-Lung Preparation.—Electrical stimulation of those ganglia of the sympathetic chains which lie below the stellate ganglia and above the upper border of the body of the ninth dorsal vertebra was followed by an increase in the tidal air of the cat heart-lung preparation. This evidence of a decrease in bronchiolar resistance appeared 15 to 20 sec. after the onset of stimulation, and was only rarely accompanied by any significant change in the performance of the heart before blood, which had passed through the preparation during stimulation of the chains, had begun to re-enter the heart from the venous reservoir (Fig. 1). In occasional

TABLE I

R_F VALUES OF REFERENCE AMINES COMPARED AFTER EXTRACTION FROM PLASMA AND EXTRACTION FROM WATER IN THREE DIFFERENT SOLVENTS

Amine	R_F Values*					
	Phenol—0·1N-HCl		n-Butanol—0·5N-HCl		n-Butanol—16·7% Acetic v/v	
	Water	Plasma	Water	Plasma	Water	Plasma
Noradrenaline	0·20±0·02 (5)	0·21±0·03 (5)	0·11±0·08 (4)	0·12±0·07 (4)	0·25±0·08 (4)	0·26±0·07 (4)
Adrenaline	0·49±0·04 (5)	0·52±0·05 (5)	0·16±0·05 (4)	0·18±0·03 (4)	0·32±0·09 (4)	0·36±0·10 (4)
Isoprenaline	0·67±0·05 (5)	0·71±0·08 (5)	0·34±0·06 (4)	0·39±0·09 (4)	0·57±0·12 (4)	0·63±0·08 (4)

* Mean R_F values are given with standard error. The number of experiments shown in brackets.

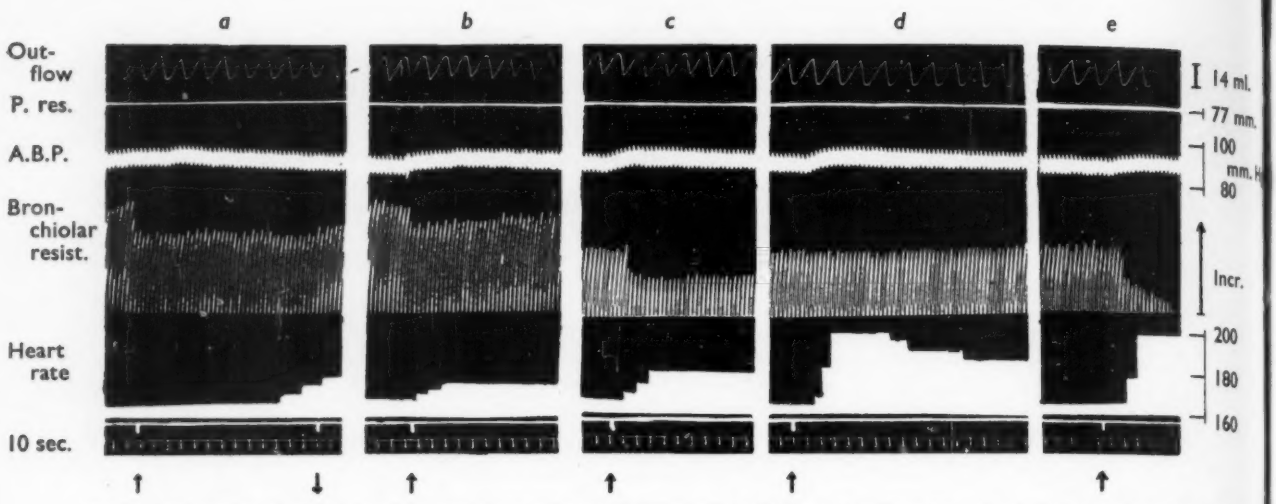

FIG. 1.—Comparison of the effects of the pulmonary sympathetic transmitter with those of isoprenaline and adrenaline in the cat heart-lung preparation. Records show, from above downwards, systemic outflow, peripheral resistance, mean brachiocephalic pressure (A.B.P.), bronchiolar resistance, heart rate, signal. Temp. 38° C. *a*, Effect of bilateral stimulation (between the arrows) of the sympathetic chains from which the nervi accelerantes have been divided. Blood was allowed to recirculate. Between *a* and *b*, this stimulation was repeated; blood passing through the preparation during this stimulation was collected in a separate reservoir. *b* shows the change (at arrow) from normal blood to blood collected during stimulation. *c*, 2 μg. (±)-isoprenaline. *d*, 4 μg. (−)-adrenaline. *e*, 3 μg. (±)-isoprenaline, each added to the venous reservoir at arrows.

preparations, however, mild tachycardia, associated with some decrease in heart size and a slight fall in right atrial pressure, was seen 30 sec. after the beginning of stimulation. Such preparations were discarded for the study of the predominant transmitter released by stimulation of the chains, because they were not typical. Their incidence may perhaps be related to the observation of Cannon, Lewis, and Button (1926) that bilateral removal of the stellate ganglia is insufficient, at least in some cats, to cause complete sympathetic denervation of the heart.

The Site at which Transmitter was Released in the Cat's Heart-Lung Preparation in Response to Stimulation of the Chains.—A series of experiments was carried out in which simultaneous serial blood samples were withdrawn from the brachiocephalic arterial cannula, from a pulmonary vein, and from the coronary sinus during stimulation of the chains. The brachiocephalic sample was collected in a syringe which was allowed slowly and continuously to fill with blood through a needle inserted through the rubber just beyond the end of this cannula. The other two samples collected were outflows from polythene cannulae. One of these had been inserted into a left upper lobe vein pointing toward the lung. The other cannula had been passed through the right auricular appendage, and its tip extended for about 1 cm. within the coronary sinus. Plasma was separated from each blood sample without delay and was tested for adrenaline-like activity on the rat uterus by the method of Gaddum, Peart, and Vogt (1949).

The results of these experiments are summarized in Table II, Expts. 1, 2, and 3. Cols. 5, 6, and 7 in Table II show the concentrations of (±)-isoprenaline in μg./ml. which were equally effective with the plasma samples in inhibiting the responses of the rat uterus to acetylcholine. The periods during which these plasma samples were collected are found in col. 2 measured in sec. from the start of stimulation. It can be seen that transmitter appeared in the pulmonary venous blood some 15 to 20 sec. after the beginning of chain stimulation (Table II, Expts. 1, 2, 3, and 6). Only after a lag period of 6 or more sec. did a measurable concentration of transmitter appear in the brachiocephalic sample. The cause of this delay is indicated by reference to cols. 3 and 4 (Table II). Col. 3 shows that the systemic output of these hearts was low; it had been deliberately reduced in order that the concentrations of transmitter in the plasma samples should be great enough for measurement. Col. 4 gives minimum estimated values for the dead space between the pulmonary veins and the point of collection beyond the brachiocephalic cannula. Mixing of pulmonary venous blood with the contents of this dead space may have delayed the arrival of measurable amounts of amine in the brachiocephalic sample. This hypothesis was tested by making one altera-

TABLE II

CONCENTRATIONS OF SYMPATHETIC TRANSMITTER IN PLASMA, DURING STIMULATION OF THE THORACIC SYMPATHETIC CHAINS, ASSAYED ON THE RAT UTERUS IN TERMS OF μG. (±)-ISOPRENALINE/ML. PLASMA

Expt. No.	Sampling Period. Sec. from Onset of Stimulation	Systemic Output ml./min.	Minimum Estimate of Innominate Dead-space (ml.)	Concentration of Transmitter Expressed as μg. (±)-Isoprenaline/ml. Plasma		
				Pulm. Vein	Innom. Art	Coronary Sinus
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	0–13	33	4·5	Trace	Nil	Nil
	13–24	33		0·08	Trace	,,
2	0–15	40	4·0	Trace	,,	Trace
	15–25			0·07	0·05	0·03
3	0–12	38	5·5	Nil	Nil	Nil
	12–20			0·065	,,	,,
4	120–160	46	—	0·125	0·110	0·085
5	20–40	54	3·0	0·08	0·05	0·03
	120–150			0·13	0·12	0·08
6	10–15	36	2·0	0·04	0·03	Nil
	15–20			0·07	0·06	Trace

tion in technique. The brachiocephalic sample was collected through a fine polythene cannula which passed through the tip of the brachiocephalic cannula (Table II, Expt. 5) and on into the aorta to open a little above the aortic valves (Table II, Expt. 6). The results confirm the hypothesis. Reduction in the dead space between the pulmonary veins and the point of brachiocephalic sampling greatly reduced the delay in the appearance of transmitter in the latter sample.

Whereas, after 2 min. of chain stimulation, the concentration of transmitter in the brachiocephalic sample did not differ significantly from that in the pulmonary venous sample, that in the coronary sinus sample was lower. This fact, coupled with the slow onset of tachycardia during stimulation of the chains (Fig. 1a), indicated partial destruction of the transmitter during circulation through the heart.

The results from four other experiments have been excluded from Table II because unidentified stimulant activity in the plasma, especially in that from coronary sinus blood, made the estimation of sympathomimetic activity in untreated plasma impossible.

The experiments described above proved that, in cats, stimulation of the sympathetic chains caused the liberation of an adrenaline-like substance into the pulmonary circulation. That this substance originated from postganglionic nerve fibres was established by the use of choline 2:6-xylyl ether bromide (TM10) provided by Professor W. A. Bain for this purpose. TM10 has been shown selectively to prevent the liberation of transmitter from postganglionic adrenergic nerve fibres (Exley, 1956; Bain and Fielden, 1956). Since the action of TM10 takes 30 to 35 min. to develop, and as the effects of stimulation of the sympathetic chains on tidal air cannot be shown with certainty for more than 45 min. after ligature of the aorta, TM10 was added to the blood in the external reservoir just before the external heart-lung circuit was established. Fig. 2 shows that TM10, so used, blocked concurrently the effects both of stimulation of the peripheral ends of the nervi accelerantes on the heart, and the effects of chain stimulation on the bronchiolar resistance, without affecting the actions of adrenaline or isoprenaline, and without blocking the effects of vagal stimulation.

Anatomical evidence indicates that the postganglionic sympathetic fibres to the smooth muscle of the bronchi must be considered the most probable source of the amine liberated into the pulmonary veins of the cat on stimulation of the sympathetic chains. The normal drainage of most of the bronchial venous blood is known to be by way of the pulmonary veins in this species. There is, however, commonly a very small additional bronchial vein on the right, opening into the azygos vein near the heart, and, less commonly, another still smaller bronchial vein on the left, opening into the superior intercostal. The combined flow was therefore measured in an animal in which both these veins were present; with the chest open, during positive-pressure ventilation, the total flow was less than 4 ml./hr. The ligature of these two accessory bronchial veins, and of all other minute twigs entering or leaving the great vessels, was a routine practice during the construction of the heart-lung preparations, and was not attended by change in tidal air. In the final preparations, the reservoir volumes decreased by less than 6 ml./hr., and the chest cavities remained

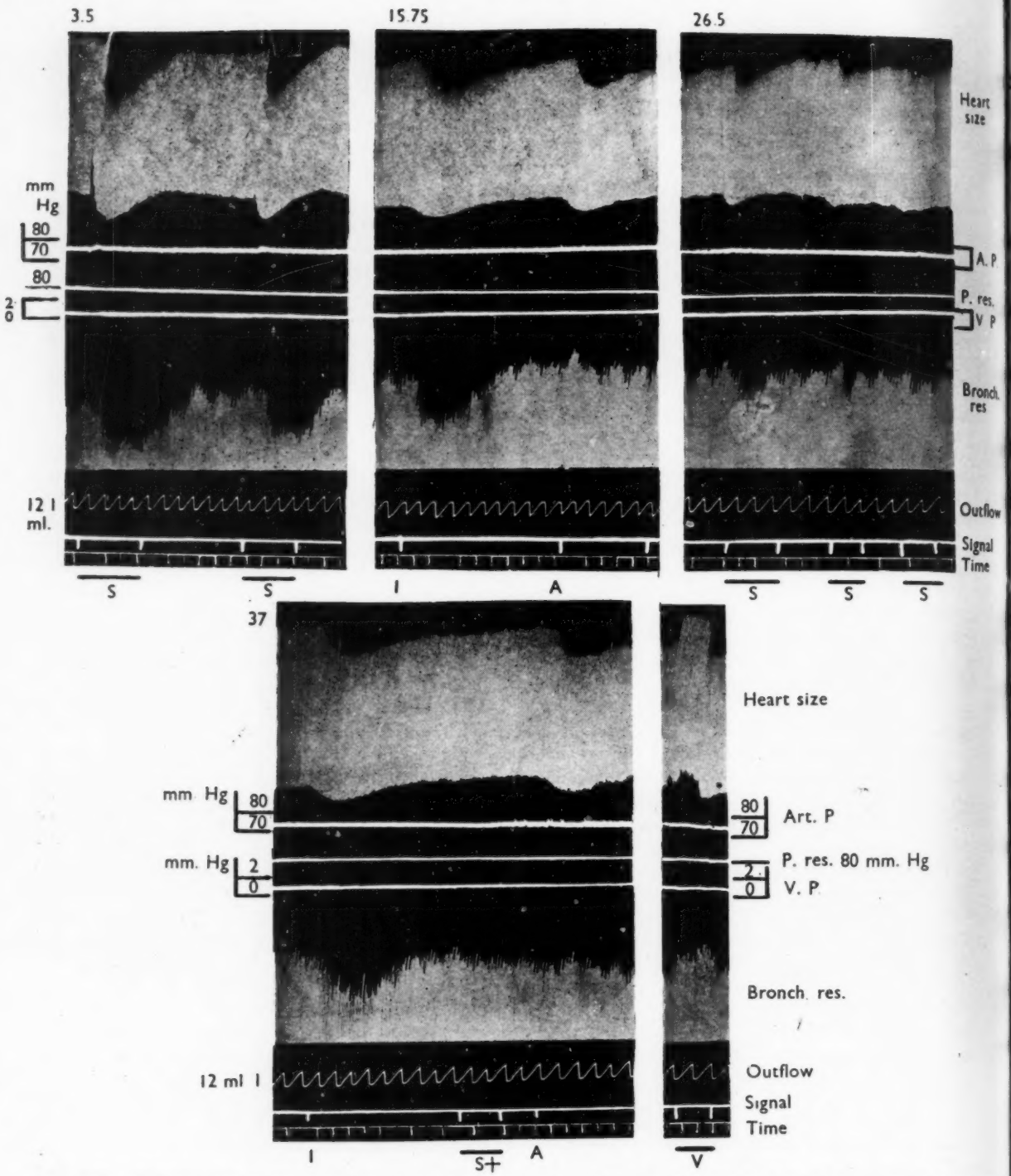

FIG. 2.—Records from a cat heart-lung preparation in which the right nervi accelerantes had been divided; the left were still attached to the sympathetic chain. TM10, which blocks postganglionic transmission selectively in the sympathetic outflow, blocks the effects of chain stimulation and of stimulation of the nervi accelerantes concurrently. TM10, 1 mg./100 ml., and pilocarpine nitrate 75 mg./100 ml. in circuit blood. Records from above downward show heart size, mean arterial pressure, peripheral resistance, venous filling pressure, bronchiolar resistance, systemic outflow, signal, and time trace recording irregularly 30 or 60 sec. The numerals above the records denote time in min. from ligature of the aorta. S=bilateral stimulation of the sympathetic chains from just above the stellate ganglia to T8, 7 v., 250 μsec., 15/sec. I=5 μg. (±)-isoprenaline. A=5 μg. (−)-adrenaline. S+=stimulation of the chains as before but with stimulation of peripheral right nervi accelerantes (2 v., 250 μsec., 15/sec.). V=stimulation of peripheral right vagus (2 v., 250 μsec., 15/sec.). Note that the effects of adrenaline and isoprenaline added to the circulating blood are unaffected by TM10.

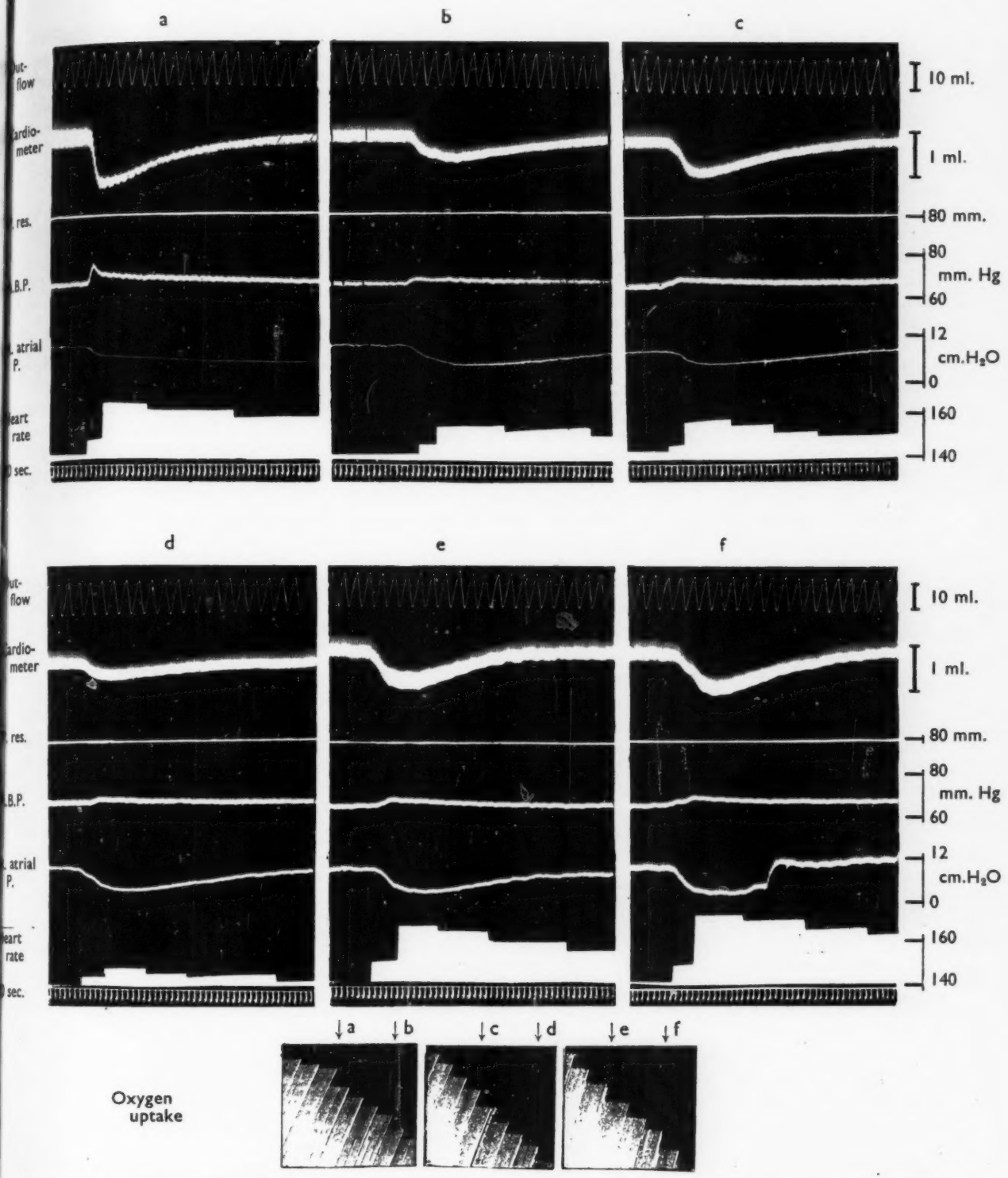

FIG. 3.—Cat heart-lung preparation. Records show, from above downwards, systemic outflow, heart size, peripheral resistance, mean arterial pressure, right atrial pressure, heart rate. Drugs were injected into the venous reservoir at *a*, 10 μg., *c*, 7.5 μg., and *d*, 5 μg. (±)-isoprenaline. *e*, 10 μg., and *f*, 20 μg. (−)-adrenaline. *b*, a change from normal blood to blood collected during chain stimulation. Below, a record of oxygen consumption: each drop in the record is an arrest of the trace for 5 min.

blood-free. The small loss in circulating volume was therefore attributed to evaporation from the lung surfaces, and it was concluded that the whole of the bronchial venous drainage had entered the pulmonary circulation.

The Pharmacological Actions of the Pulmonary Transmitter Substance. — The pharmacological activity of this transmitter was first examined in the untreated whole blood into which it had been liberated. Then the constituent amines were separated chromatographically from protein-free concentrated extracts of the plasma, and were identified by their R_F values, colour reactions, and biological activity.

The cat heart-lung preparation was chosen for both series of experiments although the sympathetic chains in this preparation only transmitted for 45 min. after ligature of the aorta. The great advantages of the preparation lay in the facts that it can be supplied with normal blood at a constant rate from the first of the two reservoirs, and blood which has passed through the preparation during stimulation of the chains can be returned to the second reservoir. This blood, collected during stimulation, can then either be withdrawn for chromatographic work, or can be fed back into the preparation for the study of its pharmacological actions after the direct effects of stimulation have worn off.

The Action of Blood Containing Pulmonary Transmitter on the Heart-Lung Preparation of the Cat.—The effects of (−)-adrenaline, (−)-noradrenaline, and (±)-isoprenaline added to normal venous blood were compared in the cat heart-lung preparation with those of blood containing transmitter. The temperature, the venous inflow, and the peripheral resistance were kept constant in these experiments. In four experiments, changes in heart rate, tidal air, right atrial pressure, and mean arterial pressure were measured. Records from a typical experiment are shown in Fig. 1. The first record, *a*, shows the effect of chain stimulation when blood passing through the preparation was allowed to recirculate; it has already been discussed. Chain stimulation was repeated between the first and second records, but the blood passing through the preparation during stimulation was collected in a separate reservoir. Record *b* shows the change from normal venous blood to the blood collected during stimulation. The change-over was almost immediately followed by a decrease in bronchiolar resistance and a moderate increase in heart rate. Record *c* shows that very similar changes were induced by the addition of 2 μg. (±)-isoprenaline to the reservoir of normal venous blood, when this was supplying the preparation. In contrast, the addition of 4 μg. (−)-adrenaline to this reservoir, record *d*, caused a much greater increase in heart rate than did the blood containing transmitter, but was without effect on the bronchiolar resistance.

Changes in heart size, heart rate, right atrial pressure and oxygen uptake were measured in five other experiments which all gave similar results. One such experiment is illustrated in Fig. 3 in which the tracings show that the changes recorded in right atrial pressure were too variable, and those in mean arterial pressure too small, to differentiate with any certainty between the actions of adrenaline and isoprenaline. The effects of these amines did, however, become clearly distinguished when graphs were plotted for each experiment relating changes in oxygen consumption and in heart size as ordinates to increase in heart rate as abscissae. Typical graphs from individual experiments are shown in Fig. 4*a* and *b*. (±)-Isoprenaline (open circles) caused both a greater increase in oxygen consumption, and a greater decrease in heart size, per unit increase in heart rate than did (−)-adrenaline (crosses). The effects of the pulmonary transmitter (dark squares) resembled much more closely those of (±)-isoprenaline (o) than those of adrenaline (x).

Noradrenaline is well known to be less effective than adrenaline in producing change in bronchiolar resistance and in oxygen consumption, but to be equally effective as adrenaline on heart rate. These facts, which were confirmed by experiment, made it very unlikely that noradrenaline would prove a major component of the pulmonary transmitter.

Chromatographic Separation of the Amines of Pulmonary Sympathetic Transmission.—Successive portions of chromatograms, prepared as described above, were eluted and tested for sympathomimetic action both on the blood pressure of the spinal cat and on the rat uterus. Eluates from areas corresponding in R_F value to noradrenaline, adrenaline, and isoprenaline were separately prepared for examination. Eluates from intervening areas were combined to give a single "blank" solution for bioassay. In two experiments this blank also represented an area of paper of R_F value greater than that of isoprenaline. Table III shows that significant sympathomimetic activity was found only in eluates from chromatographic zones which had R_F values corresponding to those of noradrenaline, adrenaline, and isoprenaline. This activity has been expressed in μg. of the reference compound of corresponding R_F value

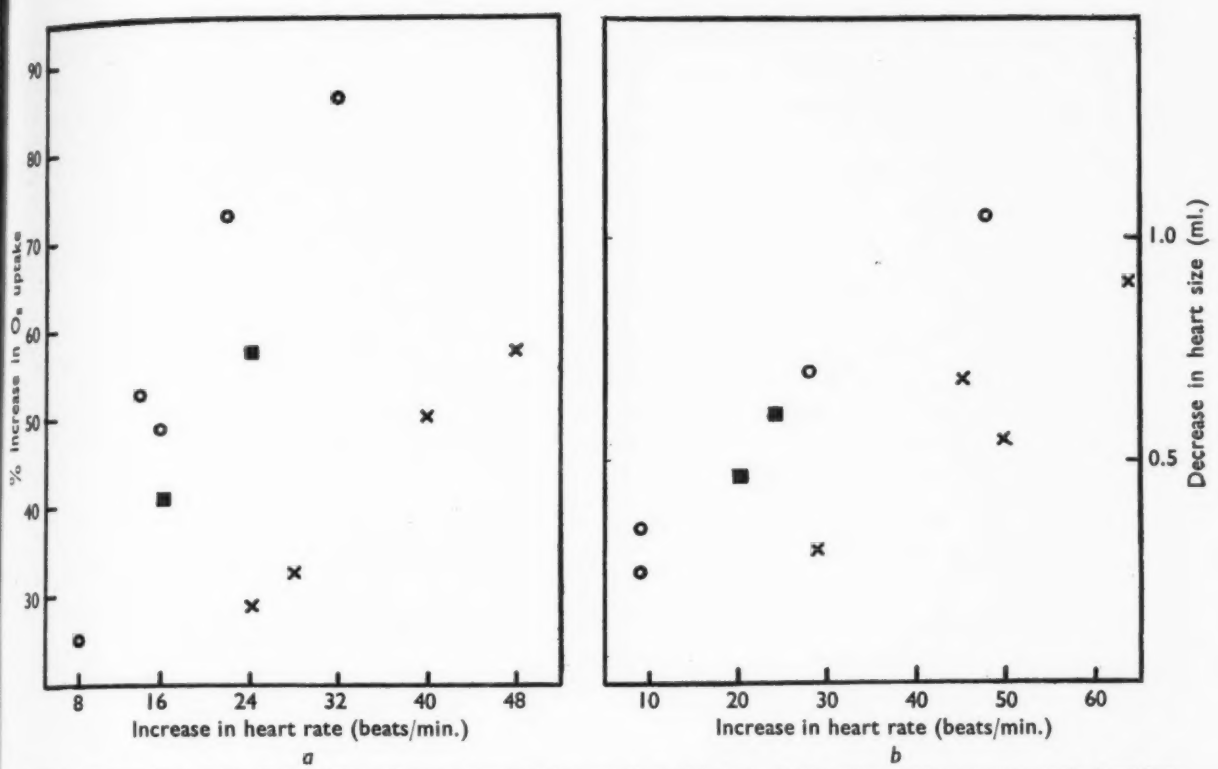

FIG. 4.—Comparison of the effects of the pulmonary sympathetic transmitter with those of adrenaline and isoprenaline on the heart-lung preparation of the cat. Each graph represents data collected in a single experiment of the type illustrated in Fig. 3. Abscissae, increase in heart rates. Ordinates, % increase in O_2 consumption (*a*), and decrease in heart size (*b*). Open circles, (±)-isoprenaline; crosses, (−)-adrenaline; and black squares, blood collected during stimulation of the sympathetic chains.

per 100 ml. plasma. (±)-Isoprenaline was used as standard for the assay of "blank" activity. Where none was demonstrated, a figure has been entered which represents the concentration of amine which might have remained undetected.

The important fact shown in Table III is the great increase of activity in the fraction with R_F value equivalent to isoprenaline that resulted from bilateral stimulation of sympathetic chains from which the nervi accelerantes had been severed. This

TABLE III

CONCENTRATIONS OF SYMPATHOMIMETIC AMINES IN THE PLASMA OF BLOOD WHICH HAD PASSED THROUGH A CAT HEART-LUNG PREPARATION IMMEDIATELY BEFORE ("C" SAMPLE) AND DURING STIMULATION OF THE THORACIC SYMPATHETIC CHAINS FROM WHICH THE NERVI ACCELERANTES HAD BEEN DIVIDED ("S" SAMPLE)

These amines were assayed after chromatographic separation in terms of the reference amine of corresponding R_F value.

Expt. No.	Chromatographic Solvent	Systemic Blood Flow (ml./min.)	Plasma Sample		Activity of Serial Chromatographic Eluates against Different Reference Compounds, by Different Assay Methods (see Text) (μg./100 ml. Plasma)				Preparation of Blood Donor
			Nature	Vol. Used (ml.)	(−)Noradrenaline	(−)Adrenaline	(±)-Isoprenaline	Blank (±)-isoprenaline	
					Cat B.P.	Rat Uterus	Rat Uterus	Rat Uterus	
1	Phenol-HCl	45	C	20	7·6	2·8	<0·3	<0·3	Spinal adrenalectomized
			S	35	7·5	3·5	30·0	<0·4	
2	,,	61	C	30	6·4	12·2	1·5	<0·5	Spinal
			S	35	6·5	12·0	9·0	<0·5	
3	,,	38	C	25	<3·5	1·0	3·0	1·0	Spinal sympathectomized, adrenalectomized
			S	40	<2·0	1·0	22·0	1·0	
4	Butanol-acetic	34	C	25	<3·0	<0·3	<0·3	<0·3	,, ,,
			S	35	<2·5	2·4	23·0	<0·8	
5	,,	43	C	27	<3·0	0·5	4·0	<0·2	,, ,,
			S	52	<1·5	3·8	18·0	<0·1	

fraction undoubtedly contained the predominant amine of the pulmonary transmitter. A much smaller, but probably significant, increase in adrenaline was observed under the same circumstances (Table III, Expts. 4 and 5), but in no case was there an increase in the noradrenaline.

The varying concentrations of adrenaline and of noradrenaline in the control plasma samples should probably be related to differences in the methods used to collect blood for these experiments (Table III, last column). Variation in the amine content of donor blood is highly likely to have been reflected in these experiments, because the time elapsing between ligature of the aorta and the completion of blood sampling was short, and varied only between 18 and 21 min. The small amounts of activity in the isoprenaline R_F fractions from control plasma (Table III, Expts. 2, 3, 5) may have been due to slight traction of the electrodes on the chains.

Since isoprenaline was used successfully as a chromatographic reference compound for the predominant amine released during stimulation of the sympathetic nerves to the lungs throughout these and all other experiments, it must be concluded that the R_F values for these two compounds did not differ significantly either with phenol-hydrochloric or with butanol-acetic as solvents.

Colour Reactions.—The predominant amine released by stimulation of the sympathetic nerves to the lungs, like isoprenaline, gave an adrenochrome reaction when treated with oxidizing reagents in weakly alkaline solution. Adrenochrome formation is typical of catechol amines with primary or secondary amino nitrogen in the side chain. The reaction product with ferricyanide was fluorescent under ultraviolet light ; this would not occur were the hydroxyl group in the side chain substituted (Lund, 1949).

The Pharmacological Actions of the Predominant Amine of Pulmonary Adrenergic Transmission.—The predominant amine released by stimulation of the adrenergic nerves to the lungs was separated chromatographically, and was then compared with isoprenaline in a series of parallel assays. In the four experiments shown in Table IV use was made of their vasodilator action in cats under chloralose anaesthesia and of their antagonism to the action of acetylcholine on the rat uterus and colon. This type of parallel assay was used for the characterization of the third amine of the adrenal gland and has already been illustrated (Lockett, 1954, Figs. 3 and 4). Throughout the present work either the uterus or the colon was taken from a strain of rats which were normally sensitive to isoprenaline, but which were abnormally insensitive to adrenaline (Lockett, 1954). Table IV shows that the amine released by stimulation of adrenergic nerves to the lungs could not be distinguished from isoprenaline, but was clearly differentiated from adrenaline by these methods.

TABLE IV

THE AMINE OF PULMONARY SYMPATHIC TRANSMISSION OF R_F VALUE EQUIVALENT TO ISOPRENALINE IS DIFFERENTIATED FROM (−)-ADRENALINE, BUT NOT FROM (±)-ISOPRENALINE, BY PARALLEL ASSAYS ON THE UTERUS AND COLON OF THE RAT, AND ON THE MEAN ARTERIAL PRESSURE OF CATS ANAESTHETIZED WITH CHLORALOSE

Expt. No.	Activity of Eluates Expressed as:					
	μg. (−)-Adrenaline/ml.			μg. (±)-Isoprenaline/ml.		
	Rat Uterus	Rat Colon	Cat B.P.	Rat Uterus	Rat Colon	Cat B.P.
1	40*	8·0	1 ml. depressor 2 μg.(−) adren. pressor	4·0*	4·0	4·0-4·5
2	3·0	12·0*	1 ml. depressor 1 μg.(−) adren. pressor	3·5	3·0*	3·0-3·5
3	5·5	30·0*	1 ml. depressor 3 μg.(−) adren. pressor	7·0	8·0*	7·0-8·0
4	26·0*	5·0	1 ml. depressor 4 μg.(−) adren. pressor	4·5*	4·0	4·0-4·5

* Tissue taken from a strain of rats normally sensitive to isoprenaline but abnormally insensitive to adrenaline.

In all subsequent experiments, eluates of the pulmonary amine were first standardized in terms of half the equally active dose of (±)-isoprenaline on rat uterus and colon. Thereafter, the assumed identity of the two amines was tested on a variety of preparations. The errors which must result from attributing the activity of (±)-isoprenaline solely to the laevorotatory component are not large (Lands, Luduena, and Tullar, 1954) and lie within the error of the rat tissue assays. The results obtained are summarized in Table V.

TABLE V

RATIOS OF THE WEIGHTS OF AMINES WHICH PROVED EQUALLY EFFECTIVE IN MODIFYING THE PERFORMANCE OF CAT HEART-LUNG PREPARATIONS

The preparations were maintained at constant peripheral resistance, systemic flow, and temperature. Weights quoted for the pulmonary amine of R_F value equivalent to isoprenaline are in terms of (±)-isoprenaline ÷ 2, and were obtained by standardization of eluates on the rat uterus and colon.

Response Measured	Ratios of Equally Effective Doses			No. of Expts.
	(−)-Adrenaline	(±)-Isoprenaline ÷2	Pulmonary Amine	
Increase in heart rate..	1·0	0·27–0·45	0·27–0·40	5
Decrease in heart size	1·0	0·50–0·67	0·50–0·67	5
Bronchiolar dilation ..	1·0	0·12–0·20	0·12–0·18	5
Increase in O_2 uptake (driven hearts 210 beats/min.)	1·0	0·33–0·50	0·33–0·50	4

The heart-lung preparations (Table V) were all maintained at constant temperature, systemic output, and peripheral resistance. In the first five experiments, pilocarpine nitrate, 50 μg./100 ml., was added to the circuit blood. This caused the moderate bronchiolar constriction required, but also slowed the hearts by 8 to 14 beats/min. In these preparations, isoprenaline and the pulmonary amine equated in their actions on heart rate and size and in the relief of bronchiolar constriction. In the last four experiments in Table V, pilocarpine was omitted and the hearts were driven at constant rates which varied from 208 to 212 beats/min. The changes in oxygen consumption produced by these amines were measured; again isoprenaline and the pulmonary amine proved equally active (Table V). The latter two amines also showed great similarity in duration of action. Their effects on heart rate and on oxygen consumption lasted more than twice as long as those of adrenaline. In the normal heart-lung preparation isoprenaline and the pulmonary amine had actions qualitatively similar but quantitatively different from those of adrenaline (Table V). By contrast, after pentobarbitone, when the heart could no longer adapt to changes in venous inflow without marked change in right atrial pressure, both pulmonary amine and isoprenaline caused dilatation of the heart and a steep rise in right atrial pressure, whereas adrenaline still caused a fall in atrial pressure and a diminution of heart size.

Finally, the pulmonary amine, like isoprenaline, was shown to dilate the vessels of the rabbit ear, and to cause relaxation of the rabbit uterus, whereas adrenaline constricted the vessels of the rabbit ear and caused contraction of the rabbit uterus (Lands, 1947).

Discussion

The evidence presented shows that a catechol amine was released into the pulmonary circulation of the cat when the adrenergic fibres to the lungs were stimulated. This amine proved, after chromatographic separation, indistinguishable from isoprenaline in R_F values, colour reactions, and pharmacological activity. In addition, the actions of this separated amine very closely resembled those of total pulmonary adrenergic transmitter in blood when examined on the heart-lung preparation of the cat. This fact indicates that the chemical procedures used in the preparation of plasma extracts, in chromatography, and in elution, produced no artifact.

The pulmonary amine is not only differentiated from noradrenaline by its R_F values. Its actions on the rat uterus, the blood pressure of the cat, the rabbit ear, and the rabbit uterus are in sharp contradistinction to those of noradrenaline.

Clear differentiation of this amine from adrenaline is also evident in R_F values, in parallel assays on the cat's blood pressure, rat uterus, and rat colon (Table IV) and in actions on the cat heart-lung preparation (Table V). The values obtained for the relative potency of adrenaline and isoprenaline on heart rate, heart size, and oxygen consumption in the cat heart-lung preparation differ quantitatively from, but are similar qualitatively to, values obtained for the cat heart perfused through the coronary vessels with oxygenated salt solution (Marsh, Pelletier and Ross, 1948). This discrepancy is almost certainly to be related to the wide divergence of the experimental conditions.

The type of action shown by the pulmonary amine resembles, in general, that of noradrenaline with a single *N* substituent of chain length equal to or exceeding three carbon atoms (Konzett, 1940b). The great potency of the pulmonary amine as an antagonist of pilocarpine-induced bronchospasm restricts the possible identification of this amine to isoprenaline (*N*-isopropylnoradrenaline) or to *N*-tertiary butyl noradrenaline amongst those amines of this series which have been fully investigated—namely, those with two, three, and four carbon atoms in single *N* substitution (Marsh *et al.*, 1948). *N*-propyl and *N*-isobutyl noradrenaline are less active in this respect (Konzett, 1940b). *N*-tertiary butyl noradrenaline has been shown twice as active as isoprenaline in accelerating the heart and in increasing the force of its contraction in Langendorf preparations of cat heart (Marsh *et al.*, 1948), but it is possible that this difference between the two amines would be decreased in the cat heart-lung preparation. R_F values for *N*-tertiary butyl noradrenaline are not available. Failure to differentiate between the pulmonary amine and isoprenaline by pharmacological means cannot therefore be regarded as proof of the identity of these amines. Such an hypothesis receives support from the identical R_F values obtained for these two substances in three different solvents.

The expenses incurred in this work were defrayed by a grant from the Medical Research Council. The apparatus used was provided by the Wellcome Trust. The electrodes used for driving hearts were a gift from the Pharmacological Laboratory, University of Oxford. Professor W. A. Bain provided [the] drug TM 10, and I gratefully acknowledge his guidance in the use of it.

References

Bain, W. A., and Fielden, R. (1956). *J. Physiol.*, **133**, 70*P*.

Cannon, W. B., Lewis, J. T., and Button, S. W. (1926). *Amer. J. Physiol.*, **77**, 326.

Crawford, T. B. (1951). *Biochem. J.*, **48**, 203.

Exley, K. A. (1956). *J. Physiol.*, **133**, 70*P*.

Gaddum, J. H., Peart, W. S., and Vogt, M. (1949). Ibid., **108**, 467.

Knowlton, F. P., and Starling, E. H. (1912). Ibid., **44**, 206.

Konzett, H. (1940a). *Arch. exp. Path. Pharmak.*, **197**, 27.

—— (1940b). Ibid., **197**, 41.

Lands, A. M. (1947). *J. Pharmacol.*, **90**, 110.

—— Luduena, F. P., and Tullar, B. F. (1954). Ibid., **111**, 469.

Lockett, M. F. (1954). *Brit. J. Pharmacol.*, **9**, 498.

Lund, A. (1949). *Acta pharm. tox., Kbh.*, **5**, 121.

Marsh, D. F., Pelletier, M. H., and Ross, C. A. (1948). *J. Pharmacol.*, **92**, 108.

Shepherd, D. M., and West, G. B. (1953). *J. Physiol.*, **120**, 15.

Vogt, M. (1952). *Brit. J. Pharmacol.*, **7**, 325.

Brit. J. Pharmacol. (1957), **12**, 97.

ANALGESIC ACTIVITY OF DIPIPANONE HYDROCHLORIDE IN STUDENT VOLUNTEERS

BY

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(RECEIVED OCTOBER 18, 1956)

Dipipanone hydrochloride raised the threshold to ischaemic pain in healthy human volunteers. The lowest dose producing a significant rise in pain threshold was 10.0 mg. The peak effect for all doses was reached after about 2 hr. Side effects, the most common of which were drowsiness, nausea, and vertigo, are described and analysed. The drug was shown to be a histamine liberator and to cause pain and tenderness at injection sites.

During a clinical trial of dipipanone hydrochloride as an analgesic, it appeared that the doses used, 25.0 and 37.5 mg., might be at the top of the dose-response curve. It was, therefore, decided to try to construct a dose-response curve for the drug, using healthy human volunteers.

METHODS

Selection of Subjects.—The proposed experiment was explained to fourth-year medical students, and volunteers were asked to write their names on a sheet of paper passed round the class. No reward was promised. Twenty-three students offered to take part in the experiment, and three more subjects were obtained from the staff of this department.

Doses.—These were selected to form a logarithmic series which would, in the light of experience gained from the clinical trial, be likely to give at least two doses at the top of the dose-response curve. The doses selected were 4.4, 6.7, 10.0, 15.0, 22.5, and 33.75 mg. Since it was known that the drug was painful when given by subcutaneous injection, 0.5 N-saline was used as a control rather than normal saline. The subjects were unaware that an "inert" control was being used.

Allocation of Doses.—Each dose was randomly allotted a code letter, and the names of the subjects were written down in random order. The dose each subject was to receive on a given day of the experiment was allotted according to the elements of a completely randomized Latin square.

Administration of Drugs.—All doses were administered by subcutaneous injection in a volume of 1.0 ml. The ampoules were identical in appearance, and the volunteers were unaware of the dose given or of the code letters allocated to the doses.

Procedure.—The subjects attended the laboratory in small groups on days convenient to them. The only limitation imposed upon their attendance was that at least 48 hr. were allowed to elapse between injections.

The analgesic activity of the drug was assessed by a method based upon that described by Keele (1952). Each subject was provided with a sphygmomanometer and a spare sphygmomanometer bulb. The cuff was placed around the upper arm—the right arm if the subject was right-handed, the left if left-handed—and inflated to a pressure of 220 mm. of mercury to occlude both venous and arterial circulations. In time to a neon timer which gave an audible pulse every second the subject squeezed the spare bulb with the hand to which the circulation was occluded. The number of squeezes necessary to produce pain in the forearm was noted; the subject then continued squeezing until the pain was, in his or her estimation, moderate. This second reading was also recorded, and the mean of these two readings was taken as an index of pain threshold.

Following a control observation, the drug was injected and further readings were taken at 10-min. intervals during the first hour, and at hourly intervals thereafter, up to a total of 8 hr. after the injection. One full series of observations was made before starting the experiments in order to give the subjects experience in the method.

After injection of the drug, and, to a less extent, after the injection of saline, the number of contractions necessary to produce pain increased. This increase was taken as a measure of the rise in pain threshold following injection, and was called the response. No metameter was used, the response being simply recorded as the difference between the number of contractions necessary to produce pain, as described above, before and after injection.

Each subject used the same spare bulb throughout each day's work, and care was taken to ensure that the bulb was held in the same position in the hand for all observations.

A space was provided on the record sheet in which subjects could note side effects.

Results

Of twenty-six subjects, two were hypersensitive to the drug ; their results have been excluded since it was impossible to assess them. One was unable to reach pain threshold after any dose above 6.7 mg. and experienced generalized pruritus on all doses, with severe nausea and vomiting on the two highest doses. The other became very euphoric and garrulous even on the lowest dose. The two highest doses caused severe nausea and vomiting, and on the highest dose he was disorientated, ataxic and so prostrate with vomiting that he was unable to carry out the experiment after the first 20 min.

The exclusion of these two subjects meant that the Latin square design of the experiment broke down and could not be reconstructed. It has not, therefore, been possible to obtain any information on tolerance either to the drug or to the experimental method.

Nine mean observations out of a total of 2,352 have had to be obtained by extrapolation because, at the times in question, the subjects involved were unable to reach pain threshold. These nine observations were distributed among three subjects as shown in Table I.

Table II shows that by 4 hr. after injection analgesia had passed its mean peak for the group. The analgesic effect of each dose was therefore estimated by taking the highest individual response for each subject in the first 4 hr. after injection. Thus a very few freak readings occurring several hours after injection were excluded.

Table III gives the results of the analysis of variance of the data obtained from the experiments, excluding the saline control, and shows that: (1) There is no evidence that the dose-response relationship over the range of doses used is other than linear, (2) there is a highly significant difference in response both between subjects and between doses, and (3) increasing the dose of dipipanone hydrochloride increases the ability of the drug to raise the pain threshold.

The equation expressing the relationship between dose and response is given in Fig. 1, which

Table I

DISTRIBUTION OF NINE OCCASIONS ON WHICH SUBJECTS WERE UNABLE TO REACH PAIN THRESHOLD AFTER SUBCUTANEOUS INJECTION OF DIPIPANONE HYDROCHLORIDE

Subject	Dose (mg.)	Observations	
		No.	Times
A. S.♀	22·5	5	2, 3, 4, 5, 6 hr.
J. M. Bk. ..	33·75	3	40, 50, 60 min.
J. G. D.	33·75	1	3 hr.

Table II

MEAN OBSERVED RESPONSES FOR ALL SUBJECTS UP TO 5 HR. AFTER INJECTION OF DIPIPANONE HYDROCHLORIDE

The response is the difference in the number of contractions necessary to produce pain before and after injection.

Dose	Response at				
	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
0·5 N-saline ..	11	10	8	7	5
4·4 mg. dipipanone	12	12	10	8	7
6·7 „ „ ..	11	13	12	9	7
10·0 „ „ ..	13	13	13	11	11
15·0 „ „ ..	14	16	14	12	11
22·5 „ „ ..	19	24	21	21	17
33·75„ „ ..	24	24	23	18	15

Table III

ANALYSIS OF VARIANCE OF THE RESULTS OF EXPERIMENTS TO SHOW THE ABILITY OF DIPIPANONE HYDROCHLORIDE TO RAISE THE THRESHOLD TO ISCHAEMIC PAIN IN HUMAN VOLUNTEERS

Item	SS.	d.f.	MS	VR	P
Regression ..	4,195·84	1	4,195·84	40·60	<0·01
Linearity ..	128·20	4	32·05	—	—
Doses ..	4,324·04	5	864·81	8·368	<0·001
Subjects ..	11,436·16	23	497·22	4·811	<0·001
Error ..	11,884·13	115	103·34	—	—
Total ..	27,644·33	143	—	—	—

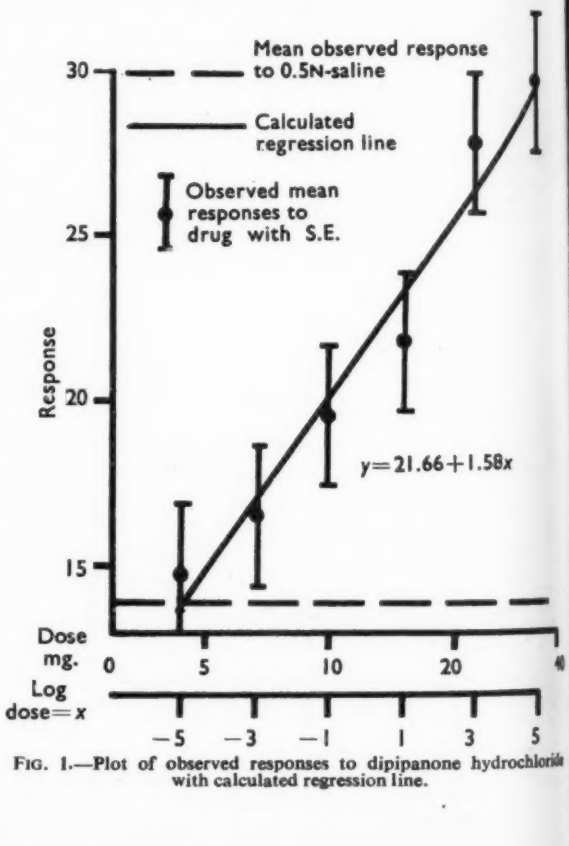

FIG. 1.—Plot of observed responses to dipipanone hydrochloride with calculated regression line.

also shows the mean observed responses to each dose with the calculated regression line.

Because there was an apparent analgesic response to 0.5N-saline, it was of interest to determine whether the variance in "response" to saline was similar to that observed with the drug. From the data obtained by experiment the variance of "responses" to 0.5N-saline is 68.78; there is no significant difference between this and the variance of response to the drug. The mean "response" to 0.5N-saline is shown in Fig. 1.

Having established this lack of significant difference in the variance of response between 0.5N-saline and the drug, the lowest dose of dipipanone hydrochloride in the series which would cause a significant rise in pain threshold was determined. As shown in Table IV, a dose of 10 mg. causes a rise in pain threshold which is barely significant.

TABLE IV

"*t*" TEST SHOWING THAT 10 MG. DIPIPANONE HYDROCHLORIDE PRODUCES A RISE IN PAIN THRESHOLD WHICH IS JUST SIGNIFICANT

Mean response to saline	$=13{\cdot}79 \pm 3{\cdot}71$
Calculated response to 10 mg. dipipanone HCl ..	$=20{\cdot}08 \pm 2{\cdot}12$
$t(46) = \dfrac{20{\cdot}08 - 13{\cdot}79}{\sqrt{\frac{68{\cdot}78}{23} + \frac{103{\cdot}34}{23}}}$	$=2{\cdot}298$
$P = 0{\cdot}02$–$0{\cdot}05$	

Side Effects.—The incidence of side effects is shown in Table V. Not all of these effects can be regarded as undesirable. Drowsiness, euphoria, sleep, and "detachment," for instance, are effects which enhance the value of a major analgesic.

TABLE V

INCIDENCE OF SIDE EFFECTS AFTER INJECTIONS OF 0·5 N-SALINE AND VARIOUS DOSES OF DIPIPANONE HYDROCHLORIDE

Side Effects	Dose (mg.)							Total
	Saline	4·4	6·7	10·0	15·0	22·5	33·75	
Drowsiness ..	1	3	2	7	8	14	16	51
Nausea	2	3	2	5	5	14	17	48
Dizziness ..	0	0	2	4	5	10	18	39
Euphoria ..	0	2	0	4	5	4	10	25
Muzziness ..	0	0	0	2	3	6	6	17
Vomiting ..	0	0	0	0	0	2	8	10
Headache ..	1	2	3	2	3	5	4	20
Dry mouth ..	0	0	2	0	1	5	6	14
Ataxia	0	0	1	0	1	5	6	13
Epigastric discomfort	0	1	1	3	2	0	1	8
Detachment ..	0	0	0	1	1	2	4	8
Pruritus ..	0	1	0	0	2	1	4	8
Tremors ..	0	0	0	0	0	2	5	7
"Constriction" of the throat ..	0	0	0	1	1	1	4	7
Anorexia ..	0	0	0	0	0	3	1	4
Irritability ..	0	0	0	0	0	1	1	2
Deafness ..	0	0	0	0	0	1	1	2
Sleep	0	0	0	0	0	0	1	1
Total ..	4	12	13	29	37	76	113	284

Table V shows that there is a sharp rise in side effects when the dose exceeds 15 mg. and it is felt that this imposes a limit on the dose which should be used therapeutically.

It should, however, be stressed that the subjects were healthy volunteers and that the incidence of side effects—particularly dizziness, nausea and vomiting—might be expected to be greater in such subjects than in the patients confined to bed for whom dipipanone hydrochloride would be most likely to be used. Indeed, it was noteworthy that many subjects found that on lying down these symptoms were absent or not so marked. Two subjects were nauseated in the laboratory, but only vomited when going home by bus. Therefore, although the optimum dose in subjects who were up and about was about 20 mg., in recumbent patients it might justifiably be increased to 25 mg.

The generalized pruritus of which some subjects complained was rather distressing and persisted for many hours. This effect, together with the incidence of headache and epigastric discomfort, prompted the suggestion that dipipanone might be a histamine-liberator. Intradermal injection of dipipanone hydrochloride did produce a weal and flare reaction, and the area of the weal was decreased by the previous administration of 2.5 mg. triprolidine hydrochloride by mouth.

All the injections were painful and often the pain was very persistent. Many subjects had tenderness and induration at the sites of subcutaneous injection for many weeks. Slight ulceration occurred at the sites of intradermal injections. The ulcers were slow to heal and have left pigmented scars.

The inability of some subjects to reach pain threshold on occasion was probably due in part to the hypnotic effect of the drug and not solely to its analgesic action. For instance, 50 min. after a dose of 33.75 mg. one subject was compelled by fatigue to stop squeezing the bulb after 75 squeezes although no pain had been felt. After 180 min., however, he was able to squeeze the bulb 90 times and reach pain threshold.

My thanks are due to Dr. J. G. Dare for his invaluable assistance with the analysis of the results, to Professor W. A. Bain and my colleagues for their interest and encouragement, and not least to the volunteers for their willing and cheerful co-operation. I am indebted to the Wellcome Foundation for supplies of dipipanone hydrochloride.

REFERENCE

Keele, C. A. (1952). *Analyst*, **77**, 111.

Brit. J. Pharmacol. (1957), **12**, 100.

SEASONAL VARIATIONS IN THE RESPONSE OF RATS TO THE ANTIDIURETIC HORMONE

BY

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The antidiuretic response of unanaesthetized rats to intravenous doses of vasopressin has been compared in the different seasons of the year. Significant seasonal differences in sensitivity to the hormone were found; thus the rats responded to smaller doses in spring and summer. Discrimination between doses appeared to be best in summer. Relative variability in response seemed to be least in winter. There appeared from season to season to be a concomitant variation in the same or opposite direction between sensitivity in terms of the average minimum effective dose and environmental factors such as temperature, barometric pressure and relative humidity.

One of the difficulties in antidiuretic assays on rats lies in the variation in sensitivity of test animals to intravenous injections of posterior pituitary extracts. For instance, one rat may respond with a marked inhibition of water diuresis to 5 microunits (μU.) of vasopressin per 100 g. of body weight, while another may need 100 μU./100 g. to produce a similar response using the same method of assay. The present work is an attempt to analyse factors which may have some influence on this variability in response. Our material consisted of 281 rats which received two or more doses of the standard pituitary preparation (Pitressin). All the animals were males of the same strain (Wistar); they were under the care of the same animal attendant; the number of rats per cage and the diet remained the same throughout the period of experiment. All the rats were of approximately the same age and weight. They were prepared for the assay in the same manner. Their water load was kept between 6 and 8% of their body weight, as Ginsburg and Heller (1953) have shown that changes in response attributable to differences in water load within this range are insignificant. All injections were made by the same worker and the volume of fluid injected was kept constant. The time of day at which the injections were made was varied at random.

METHODS

The procedure of Ginsburg and Heller (1953) was used for the antidiuretic assay with the modification that the bladder as well as a jugular vein was cannulated. Urine volumes were measured at 5 min. intervals. Antidiuretic responses were calculated as % antidiuresis, that is, as the difference between the rate of urine secretion in the collecting period preceding injection (V_1) and that during the two collecting periods after injection (V_2) expressed as a percentage of the pre-injection rate thus:

$$\% \text{ antidiuresis} = [V_1 - V_2)/V_1] \times 100$$

A dose producing between 30 and 40% antidiuresis was regarded as the minimum effective dose.

Pitressin (Parke, Davis and Co.) was used throughout. The pressor activity of different batches was assayed by the method of Dekanski (1952) in comparison with international standard powder. The results were in good agreement with the estimates of the manufacturers (see Ginsburg, 1956).

RESULTS

The experiments were made during a period of about 1½ years. The results in Table I are arranged according to the sensitivity of animals. In the second column, the frequency distribution of animals (numbers and %) is shown according

TABLE I

VARIATION IN SEASONAL FREQUENCY DISTRIBUTION OF MINIMUM EFFECTIVE DOSES OF PITRESSIN INJECTED INTRAVENOUSLY INTO UNANAESTHETIZED RATS

Pitressin (μU./100 g.)	Whole Yr.		Winter		Spring		Summer		Autumn	
	No.	%	No.	%	No.	%	No.	%	No.	%
3·125	16	5·7	1	1·8	6	10·5	9	8·3	0	0
6·25	22	7·8	1	1·8	5	8·8	15	13·8	1	1·7
10	39	13·9	2	3·5	5	8·8	30	27·5	2	3·5
12·5	50	17·7	7	12·3	14	24·6	14	12·8	15	25·9
25	98	34·9	21	36·8	24	42·1	33	30·3	20	34·5
50	47	16·7	25	43·9	3	5·3	4	3·7	15	25·9
100	9	3·2	0	0	0	0	4	3·7	5	8·6

to the minimum effective dose during the whole calendar year. The columns following give the frequency distributions in each of the four seasons. Fig. 1 expresses these results graphically. The two S-shaped curves for summer and spring fall close to one another, while those for autumn and winter are both displaced to the right by about the same amount. In the summer, 62% of the rats responded to 12.5 μU./100 g. or less, while in the winter only 19% did.

These frequency distributions were transformed into logarithmic distributions. Such distributions if normal or approximately so would, in their cumulative form, plot as straight lines on a log-probability grid, that is on a system of co-ordinates whose abscissa is the logarithmic variable and whose ordinate is the Gaussian integral. The distribution for the whole year, plotted on such a probability grid (Fig. 2), is linear apart from the extremes and justifies the conclusion of a log normal distribution ; those for the seasonal distributions give less satisfactory lines, probably due to the small number of animals. The position of the lines suggests that the mean dose required for response differs from season to season, whereas the slopes of the lines which indicate

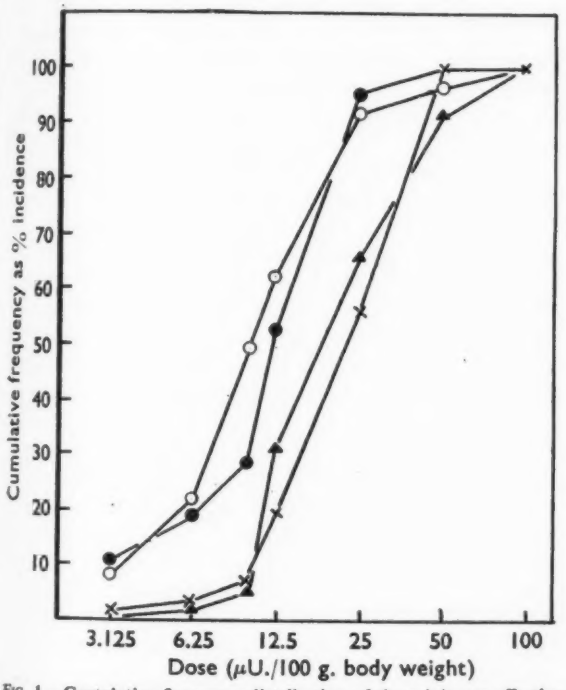

FIG. 1.—Cumulative frequency distribution of the minimum effective doses of Pitressin. Antidiuretic assays on rats. Intravenous injections. ×——× winter; ●——● spring; ○——○ summer; ▲——▲ autumn. The dose scale is logarithmic, but is graduated in true doses.

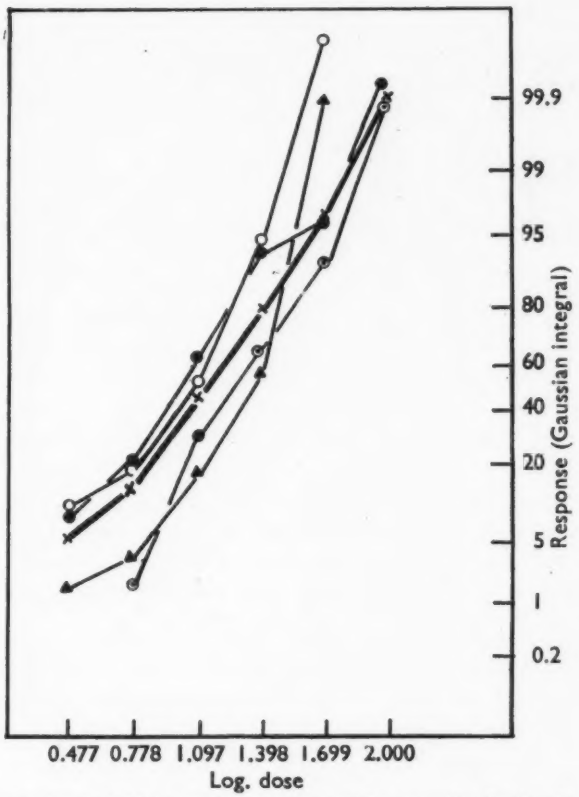

FIG. 2.—Probability graph of the seasonal frequency distributions in sensitivity. X——X whole year; ▲——▲ winter; ○——○ spring; ●——● summer; ⊙——⊙ autumn. The results of the experiments with 10 μU./100 g. (see Table I) are omitted.

the standard deviations are not very different from one another.

The arithmetic means and standard deviations of the log distributions are given in Table II. As is well known, the arithmetic mean and standard deviation of the logarithmic distribution stand in functional relationship to the corresponding statistics of the geometric distribution, the anti-log of M_{log} being the geometric mean G, and the anti-log of σ_{log} being the geometric standard deviation σ_g (Galton and MacAlister, 1879; Gaddum, 1945). These constants for the geometric distribution are shown in col. 5 and 6 of

TABLE II

LOGARITHMIC DISTRIBUTIONS (MEAN AND STANDARD DEVIATION) OF THE RESULTS SHOWN IN TABLE I

Seasons	M_{log}	σ_{log}
Winter	1·452	0·274
Spring	1·154	0·330
Summer	1·122	0·345
Autumn	1·426	0·329
Whole year	1·258	0·351

TABLE III

CHANGES IN RESPONSE AND DISCRIMINATION RELATED TO SEASONAL ENVIRONMENTAL FACTORS

1	2	3	4	5	6	7		8
	Mean Temperature (° F.)	Mean Barometric Pressure (Millibars)	Mean Relative Humidity (%)	Dose (Geometric Distribution)		Response (% Antidiuresis)		
				G	σ_g	Means $\bar{S}_1$	$\bar{S}_2$	Regression $\bar{S}_2/\bar{S}_1$
Winter ..	37·3	1,009·5	84·2	28·3	1·88	41·3	64·5	1·56
Spring ..	52·1	1,017·2	78·0	14·3	2·14	39·4	63·8	1·62
Summer	60·1	1,019·2	77·2	13·3	2·21	32·3	57·9	1·79
Autumn	46·6	1,013·5	88·1	26·7	2·13	40·5	62·3	1·54
Whole year ..				18·1	2·24			

Table III. Col. 5 shows the changes in average sensitivity with the season. It was greater in the spring and summer and smaller in the autumn and winter. The differences between the means are significant between winter and spring ($t=4.87$, $P<0.001$), and summer and autumn ($t=5.62$, $P<0.001$), but not significant between spring and summer ($t=0.56$, $P>0.5$) and autumn and winter ($t=0.48$, $P>0.6$).

The geometric standard deviations represent a much more homogeneous series than the geometric means, suggesting that the variation relative to the mean dose within a group of test animals remained similar from season to season, in spite of the different dose level required for response. This inference is drawn from the following relation between the standard deviation of the logarithmic and of the geometric distribution.

$$\sigma_{\log} = \log\sigma_g - \log G = \log (\sigma_g/G)$$

The logarithmic standard deviation is thus seen to be the logarithm of the relative standard deviation of the geometric distribution. Since the ratio of the standard deviation to the mean is known as the coefficient of variation, which is used for the purpose of comparing variability relative to the mean, similarity of the logarithmic standard deviations implies similarity of the relative variability.

However, it cannot be excluded that the series of geometric standard deviations exhibits a trend in the direction opposite to that of the means. If this could be established, it would afford a clue to a change in variability between the animals from season to season. However, only the logarithmic standard deviation for the winter appears significantly different from the other three values by Fisher's F test at the 0.05 level.

So far, the responses have not been considered quantitatively. Col. 7 and 8 of Table III show the magnitude of the response to a smaller dose (S_1) and a larger dose (S_2) in 163 animals in which S_2 was twice S_1—i.e., twice the "minimum effective dose." Col. 7 gives the mean response to each of the two doses and col. 8 the regression. The trend of the regression admits the conclusion that the variation from season to season in the responses to the larger dose was not parallel to that of the smaller dose. That is to say, discrimination between doses seemed greater in the summer and, perhaps, in the spring than in autumn and winter.

As the antidiuretic response could be related to the seasons of the year, it was felt worth while to inquire whether the response was associated with any meteorological condition. In Table III three physical characteristics of the seasons, recorded in Bristol, are given in col. 2, 3, and 4. It can be seen that the higher the mean temperature and barometric pressure the greater apparently the sensitivity to vasopressin. However, there seemed to be an inverse relationship between sensitivity and relative humidity.

DISCUSSION

A seasonal variation in the quantitative response to posterior pituitary extracts has been demonstrated in frogs (Heller, 1930; Oldham, 1936; Boyd, Mack, and Smith, 1939) and toads (Jørgensen, 1950). In both species, the water-balance-effect of a given dose is greater in summer than in winter. It would now seem that an increase in the sensitivity to vasopressin during spring and summer can also be shown in the rat, a mammalian species in which seasonal variations in sexual activity are not pronounced and in which adaptability to climatic changes is excellent. It could not be established in this investigation which of the physical characteristics of seasonal changes account for the fluctuations in sensitivity and in discrimination between doses. The results suggest that there is a relation between the degree of responsiveness to the antidiuretic hormone and factors such as environmental temperature, barometric pressure, and humidity. These associations may not be causal, but they may be taken to indicate that the influence of these factors on mammalian water metabolism and neurohypophysial function deserves further study. Such work is in progress.

Our sincere thanks are due to Mr. G. E. Clothier, of the Long Ashton Horticultural Research Station, who supplied the meteorological information.

References

Boyd, E. M., Mack, E. G., and Smith, A. E. (1939). *Amer. J. Physiol.*, **127**, 328.

Dekanski, G. (1952). *Brit. J. Pharmacol.*, **7**, 567.

Gaddum, J. H. (1945). *Nature, Lond.*, **156**, 463, 747.

Galton, F., and MacAlister, D. (1879). *Proc. Roy. Soc.*, **29**, 365.

Ginsburg, M. (1956). *Brit. J. Pharmacol.*, **11**, 245.

—— and Heller, H. (1953). *J. Endocrin.*, **9**, 267.

Heller, H. (1930). *Arch. exp. Path. Pharmak.*, **157**, 298.

Jørgensen, C. B. (1950). *Acta physiol. scand.*, **22**, Supp. 78.

Oldham, F. K. (1936). *Amer. J. Physiol.*, **115**, 275.

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THE EFFECT OF ETHER AND PENTOBARBITONE SODIUM ON GASTROINTESTINAL FUNCTION IN THE INTACT RAT

BY

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The effect of ether and pentobarbitone anaesthesia on gastrointestinal motility and absorption has been studied by measuring simultaneously gastric emptying, small intestinal transit and intestinal absorption of glucose and iodide in intact rats. Both gastric emptying and intestinal transit are very slow under ether anaesthesia, but with pentobarbitone there is no significant delay. The absorption of glucose by the small intestine is significantly impaired by ether but not by pentobarbitone, and a tracer dose of iodide is absorbed normally under both forms of anaesthesia.

Many experiments on gastrointestinal function in animals have been carried out under anaesthesia, but there has been little quantitative work on the effect of anaesthetics on the gastrointestinal tract. Gastric emptying has been studied radiologically after opaque meals (Cannon and Murphy, 1907; Baron and Barsony, 1914; Sleeth and van Liere, 1938; van Liere and Northup, 1941) and pressure changes in the stomach and intestine have been recorded from balloons placed in the stomach or in Thiry-Vella loops (Miller, 1926; Quigley, Barlow and Himmelsbach, 1934; Bisgard and Johnson, 1939; Burstein, 1939; Gruber and Gruber, 1941; Golden and Mann, 1943). Intestinal motility has also been investigated by measuring the distance travelled by a charcoal-acacia meal (Emerson, 1937; van Liere, Stickney and Northup, 1947). The usual experimental animal has been the dog. All authors find some delay in gastric emptying and intestinal motility under ether anaesthesia. Barbiturates have sometimes been said to decrease gastrointestinal motility, but other authors have found little effect.

There is little convincing evidence available on the effect of anaesthetics on intestinal absorption. Some techniques are open to the objection that variations in gastric emptying are not taken into consideration (Heller and Smirk, 1932) and others to the objection that unanaesthetized controls are not available (Valette and Anglade, 1953). Cordier and Chanel (1948) found delayed intestinal absorption of glucose after both thiopentone and ether, but their work is open to the criticism that they estimated gastric emptying from residual gastric glucose, ignoring gastric absorption of glucose (Reynell and Spray, 1956a). Olmsted and Giragossintz (1929) found flat glucose tolerance curves in dogs anaesthetized with amylobarbitone, and these could not be entirely accounted for by delayed gastric emptying.

We have investigated the effects of ether and pentobarbitone sodium on gastrointestinal function using a method by which gastrointestinal absorption and transit can be measured simultaneously in the intact rat (Reynell and Spray, 1956a, b).

METHODS

Adult male rats of the Wistar strain (200 to 400 g. weight) were used. They were starved overnight but allowed water *ad libitum*, and on the following morning each rat was intubated with a gum elastic catheter and 4 ml. of a solution, containing approximately 3 mg. phenol red, 800 mg. glucose, and 2 μc. ^{131}I as sodium iodide of high specific activity, were introduced into the stomach. The animals were divided into the following groups:

Intraperitoneal Pentobarbitone Sodium.—Seven rats were given 12 mg. pentobarbitone sodium in 0.2 ml. by intraperitoneal injection. Anaesthesia was achieved within a few minutes and maintained for 2 hr., after which time the animal was killed. It was usually necessary to give a further injection of 6 mg. pentobarbitone sodium after about 90 min. to maintain full surgical anaesthesia.

Oral Pentobarbitone Sodium.—Six rats were given 24 to 36 mg. pentobarbitone sodium with the glucose and phenol red. In all but one of them this induced

anaesthesia within 10 min., which persisted for 2 hr., when the animals were killed. A further four rats (not included in the series) died under anaesthesia.

Ether.—Twelve rats were kept anaesthetized by open ether for 2 hr. after intubation and were then killed.

Controls.—Nine rats were not anaesthetized but were killed 2 hr. after intubation.

The stomach and small intestine of each rat were divided into the following four segments: stomach, proximal half of small intestine, third quarter of small intestine, and distal quarter of small intestine. Each segment was homogenized and analysed separately for phenol red, glucose, and ^{131}I by the methods of Reynell and Spray (1956a).

Gastric emptying, intestinal transit for each segment of small intestine, estimated intestinal absorption of glucose and of iodide, and % intestinal absorption of glucose and of iodide were calculated as described elsewhere (Reynell and Spray, 1956a, b).

Extent of Gastric Emptying.—This was the amount of phenol red which had left the stomach during the experimental period. It was calculated by expressing the amount of phenol red recovered from the stomach at the end of the experiment as % of the amount recovered from the whole gastrointestinal tract.

Intestinal Transit.—Transit through a segment of small intestine was calculated by measuring the amount of phenol red which had entered the segment during the experimental period and the amount which had passed on to more distal segments during the same period of time. The latter was then expressed as % of the former.

Estimated Intestinal Absorption.—The absorption of glucose or of iodide was found by calculating the amount of test substance which had left the stomach during the experimental period (calculated from the dose given and the gastric emptying) and then subtracting the amount finally recovered from the small intestine.

% Intestinal Absorption.—For this quantity, the estimated intestinal absorption of glucose or of iodide was expressed as % of the amount of test substance which had left the stomach during the experimental period.

Results

Gastric Emptying and Intestinal Transit

These results are given in Table I. There were no statistically significant changes in motility under pentobarbitone anaesthesia, although both mean gastric emptying and intestinal transit were slightly slower than in the controls, particularly when the drug was given by intraperitoneal injection. Since these differences were not significant, no attempt was made to assess the effect of inert intraperitoneal injections. Under ether anaesthesia, on the other hand, gastric emptying and intestinal transit were significantly delayed. In no animal did more than 34% of the initial dose of phenol red leave the stomach in 2 hr. In 4 of 12 animals no phenol red had passed beyond the first half of the small intestine, and in only 3 had any entered the distal quarter of the small intestine during the 2 hr. following intubation. In all controls, some phenol red had entered the distal quarter of the small intestine and in 3 some had entered the caecum by the time the animal was killed.

TABLE I

EFFECT OF PENTOBARBITONE SODIUM AND OF ETHER ON GASTRIC EMPTYING AND TRANSIT THROUGH THE SMALL INTESTINE

	No. of Animals	% Gastric Emptying	% Intestinal Transit		
			1st Half	3rd Quarter	4th Quarter
Normal controls	9	66·0 (±5·8)	90·0 (±1·5)	45·6 (±14·1)	12·9 (±9·6)
Pentobarbitone (oral)	6	62·0 (±9·0)	86·0 (±3·2)	55·7 (±7·1)	4·3 (±4·4)
Pentobarbitone (i.p.)	7	53·7 (±5·4)	84.3 (±1·9)	34·3 (±10·7)	0
Ether ..	12	21·3 (±3·2)	35·4 (±9·9)	12·0 (±7·3)	0

The numerals in brackets give the standard error of the mean.

Absorption of Glucose and Iodide

Under pentobarbitone anaesthesia, oral or intraperitoneal, the amount of glucose and iodide absorbed and the % intestinal absorption of each were slightly reduced as compared with controls, but the differences were not statistically significant. The reduction in the amount absorbed was in part due to the rather longer mean gastric emptying time in the anaesthetized animals. Under ether anaesthesia, the amounts of glucose and iodide absorbed were much reduced, but this was due mainly to the delayed gastric emptying. Nevertheless there was a significant reduction (difference/S.E. difference = 3.48) in % intestinal absorption of glucose. The percentage intestinal absorption of iodide was not significantly reduced. Table II records these results.

TABLE II

ABSORPTION OF GLUCOSE AND OF IODIDE IN TWO HOURS BY THE SMALL INTESTINES OF NORMAL AND ANAESTHETIZED RATS

	No. of Animals	Estimated Intestinal Absorption of Glucose (mg.)	% Intestinal Absorption of Glucose	Estimated Intestinal Absorption of Iodide (% of Dose Given)	% Intestinal Absorption of Iodide
Normal controls	9	502·4 (±41·2)	95·8 (±1·1)	59·7 (±5·4)	90·9 (±1·7)
Pentobarbitone (oral) ..	6	469·5 (±76·5)	94·8 (±1·2)	56·0 (±8·3)	89·3 (±1·3)
Pentobarbitone(i.p.)	7	365·4 (±34·5)	89·6 (±2·0)	48·4 (±5·5)	90·0 (±2·0)
Ether ..	12	148·5 (±27)	72·8 (±6·9)	17·8 (±2·8)	83·0 (±2·2)

The numerals in brackets give the standard error of the mean.

Discussion

We have confirmed previous qualitative observations that the emptying time of the stomach and the propulsive motility of the small intestine are much reduced by ether anaesthesia. The capacity of the small intestine to absorb glucose is also impaired. The results do not indicate the mechanism by which these changes are brought about, but Miller (1926) has shown that ether reduces the force and amplitude of intestinal contractions in a denervated Thiry-Vella loop, suggesting that it has a direct peripheral effect on smooth muscle.

We have found slightly delayed gastric emptying and intestinal transit in animals anaesthetized with pentobarbitone sodium, but the results are not significant and the changes can be of little practical importance. Although previous observations on the effect of barbiturates on gastrointestinal motility have been contradictory, some of the differences may have been due to the rate and route of administration. In a careful series of experiments Golden and Mann (1943) showed that a rapid intravenous injection of thiopentone caused transient inhibition of small intestinal motility, but there was no appreciable effect if the drug was given slowly.

We suggest that valid observations on gastrointestinal absorption and motility can be made on anaesthetized animals if pentobarbitone sodium is used, but that ether should be avoided for experiments of this kind, in which anaesthesia is prolonged.

In surgical practice it is recognized that postoperative vomiting and other gastrointestinal disturbances are commoner after ether than after other anaesthetics (Bisgard and Johnson, 1939), and, although operative trauma probably has a greater inhibitory effect on gastrointestinal motility than ether anaesthesia alone (Cannon and Murphy, 1907), ether anaesthesia might summate with other influences in precipitating paralytic ileus.

References

Baron, A., and Barsony, T. (1914). *Pflüg. Arch. ges. Physiol.*, **158**, 464.

Bisgard, J. D., and Johnson, E. K. (1939). *Ann. Surg.*, **110**, 802.

Burstein, C. L. (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 122.

Cannon, W. B., and Murphy, F. T. (1907). *J. Amer. med. Ass.*, **49**, 840.

Cordier, D., and Chanel, J. (1948). *C.R. Soc. Biol., Paris*, **142**, 1120.

Emerson, G. A. (1937). *Amer. J. digest. Dis.*, **4**, 255.

Golden, R. F., and Mann, F. C. (1943). *Anesthesiology*, **4**, 577.

Gruber, C. M., and Gruber, C. M., Jr. (1941). *J. Pharmacol.*, **72**, 176.

Heller, H., and Smirk, F. H. (1932). *J. Physiol.*, **76**, 292.

Miller, G. H. (1926). *J. Pharmacol.*, **27**, 41.

Olmsted, J. M. D., and Giragossintz, G. (1929). *Proc. Soc. exp. Biol., N.Y.*, **27**, 103.

Quigley, J. P., Barlow, O. W., and Himmelsbach, C. K. (1934). *J. Pharmacol.*, **50**, 425.

Reynell, P. C., and Spray, G. H. (1956a). *J. Physiol.*, **131**, 452.

—— —— (1956b). *Brit. J. exp. Path.*, **37**, 466.

Sleeth, C. K., and van Liere, E. J. (1938). *J. Pharmacol.*, **63**, 65.

Valette, G., and Anglade, J. (1953). *J. Physiol. Path. gén.*, **45**, 491.

van Liere, E. J., and Northup, D. W. (1941). *J. Pharmacol.*, **73**, 142.

—— Stickney, J. C., and Northup, D. W. (1947). *Gastroenterology*, **8**, 82.

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LOCAL EFFECTS AND MECHANISM OF ABSORPTION OF IRON PREPARATIONS ADMINISTERED INTRAMUSCULARLY

BY

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An attempt has been made to correlate factors involved in the absorption of iron-polysaccharide complexes administered intramuscularly. Different complexes varied greatly in degree of retention in muscle and in diffusibility in agar; these two characteristics were not closely related. The local changes in the muscle produced by the iron complexes consisted of an acute inflammatory reaction at the site of injection, with degenerative changes. Subsequent regeneration was rapid and complete. The major proportion of the absorption occurred during the initial 72 hr. and appeared to be mediated partly by the inflammatory reaction evoked, with enhancement of lymphatic transport of the iron complex. Rapid fixation by tissue macrophages impeded absorption and, with some complexes, this factor may make much of the injection inaccessible.

Intramuscular injection of drugs has been practised for over 70 years, but the factors concerned in the mechanism of absorption of the injected substances have aroused little attention. At first sight absorption from muscle would appear to involve merely a process of passive diffusion through the extracellular fluid: this process if delayed would result in formation of a depot with gradual entry of the drug into the circulation. Drinker and Field (1933) showed that the path of absorption depends upon the molecular size of the injected substance, small molecules being absorbed directly into the blood stream whilst larger ones entered the lymphatics. The molecular weight range was more precisely characterized by Barnes and Trueta (1941), who showed that venoms and toxins of molecular weight 20,000 or more are absorbed by the lymphatics whilst cobra venom of molecular weight 5,000 enters the blood stream directly. Another factor which could influence absorption is the local effect of the injected substance on the muscle, a topic on which the literature is relatively silent, though there are many reports of nerve palsies and other mishaps arising from the intramuscular administration of certain drugs. With the object of throwing some light on the mechanism of absorption of iron, a study was undertaken of a number of iron polysaccharide complexes, including commercial and laboratory preparations.

METHODS AND MATERIALS

Quantitative Study of Absorption of Iron from Muscle.—The following four preparations of iron-polysaccharide complexes were studied: the iron-dextran complex "Imferon" (IDA), an iron-dextran complex known to possess poor absorption characteristics (IDB), an iron-polysaccharide complex marketed for human use (IPH), and an iron-polysaccharide complex marketed for veterinary use (IPV).

All four preparations contained 5% (w/v) of elemental iron. Each of the iron-dextran complexes was polydisperse, but it was likely that a proportion of each lies within the molecular weight range 10,000 to 20,000. Retention of iron in muscle was studied after doses equivalent to 20 mg. Fe/kg. were injected deep into the right gluteal muscles of rabbits weighing approximately 2 kg. At varying periods of time after the injection the animals were killed, the skin was removed from the leg, and the soft tissues were dissected away from the bones and placed in an acid-washed beaker. The tissue was wet-oxidized with sulphuric and nitric acid (Middleton and Stuckey, 1954). The solution after oxidation was diluted to 250 ml., and a suitable aliquot taken for determination of iron by the thiocyanate method (Ventura and Klopper, 1951). The left (uninjected) leg was treated in a similar manner to obtain the control iron value.

In vitro *Study of the Diffusion of Iron.*—These experiments were intended to compare the rates of diffusion *in vitro* of various iron-dextran complexes prepared by different methods. Serum-agar plates were prepared containing 0.7% agar in saline and 0.01%

merthiolate, with 20% (v/v) of horse serum. The iron complex (0.1 ml.) was added to a Whatman 3MM paper disc (18 mm.) placed on the agar. After keeping at room temperature overnight, the dishes were inverted and incubated at 37° C. for 6 days. Diffusion took place symmetrically outwards from the paper disc, and usually two zones were discernible; a dark inner zone immediately around the disc, and a lighter "halo" beyond. The diameters of the total zone of diffusion and of the dark inner zone were measured.

Histological Study of the Local Changes at the Injection Site.—The four iron polysaccharide complexes used in the first experiment were studied. Each was mixed with a small amount of Indian ink to assist identification of the injection site, and 0.1 ml. containing 5 mg. of iron was injected into the left soleus muscles of 28 adult rats (112 animals in all). In the case of rats given iron-dextran complex, the right soleus muscles were injected with 0.1 ml. of 20% dextran as controls. Seven animals injected with saline were also employed as controls. The animals were killed at 1, 8, 24, and 48 hr., 1 week, 1 month, and 3 months after the injection. At autopsy the leg musculature was divided into four blocks (Fig. 1) and the histological changes in each block were studied. Formalin-fixed tissues were stained by haematoxylin and eosin and by the Prussian blue reaction, and alcohol-fixed tissues were stained also by the alcoholic periodic acid-aqueous Schiff (P.A.S.) technique (Mowry and Millican, 1953).

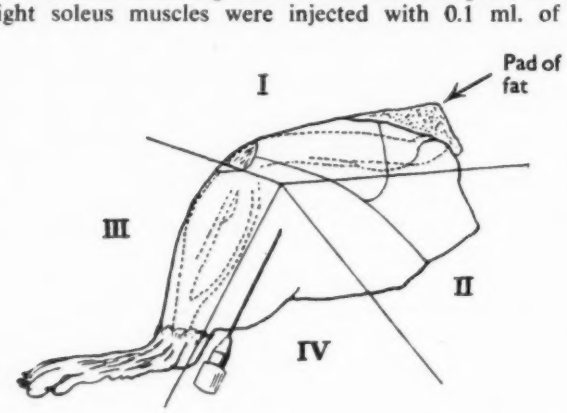


FIG. 1.—Diagram of rat hind limb showing the site of injection and the division into sectors for histological study.

RESULTS

Retention in Muscle.—The uptake of iron from the tissues at the injection site at varying times after the injection is shown in Fig. 2. Such absorption of iron as occurred from all four preparations took place largely during the 72 hr. immediately following the injection, and very little was absorbed subsequently. The iron-dextran complex IDA showed a greater initial rate of absorption and a smaller retention of iron in the muscle at 8 weeks than the other three preparations studied.

Agar Diffusion Experiments.—It was interesting to compare the above observations with measurements of the diffusion zones of the iron polysaccharide complexes in a protein-containing medium *in vitro* (Table I). There was a striking difference between the homogeneity of the diffusion zone in the case of IDA and the dark central zones of the other preparations. In the case of saccharated oxide of iron, and most probably with IPV and IPH also, the dark inner zone indicates interaction of the complex with plasma protein. The homogeneity of IDA is a reflection of the

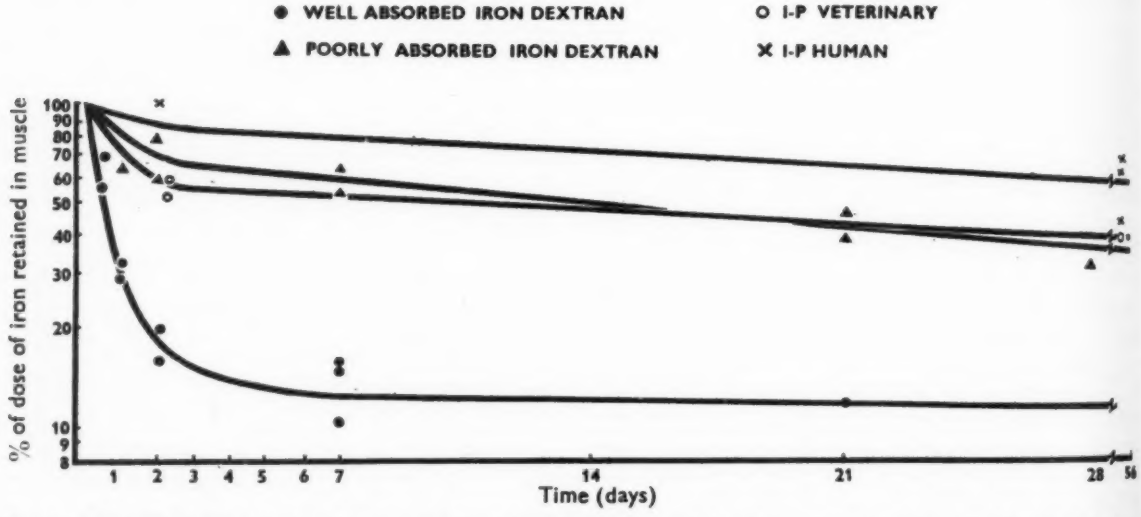


FIG. 2.—Graphs relating the retention of the iron four iron polysaccharide complexes in rabbit muscle at various times following injection.

TABLE I

IN VITRO STUDY OF DIFFUSION OF VARIOUS IRON PREPARATIONS IN SERUM-AGAR AT 37° C. FOR 6 DAYS

Iron Preparation	% Iron Content	Diameters of Diffusion Zones (mm.)	
		Total Zone	Dark Inner Zone
Saccharated oxide	2	22	22
IPV	5	32	24
IPH	5	34	26
IDA	5	37	Nil

stability of this complex (Martin, Bates, Beresford, Donaldson, McDonald, Dunlop, Sheard, London, and Twigg, 1955).

The divergence between diffusion and intramuscular absorption is seen in Fig. 3, in which % retention at the injection site at 48 hr. is compared with diffusion *in vitro* for a variety of preparations of iron-dextran. It is obvious that the diameter of the diffusion zone is not always closely correlated with the degree of absorption ; in fact, the same diffusion zone may be observed with preparations of widely differing absorption characteristics. It is clear that the determinants of intramuscular absorption include factors other than passive diffusibility.

Histological Observations. — Sections were examined of the muscles in all four sectors of the leg (Fig. 1), but significant changes were found only in area IV, where the main mass of the injection was deposited. Some seepage occurred occasionally into area II, when minor changes were seen similar to those which will be described in area IV. Little of any injection spread into areas I and III, and in no case was tissue damage seen in these.

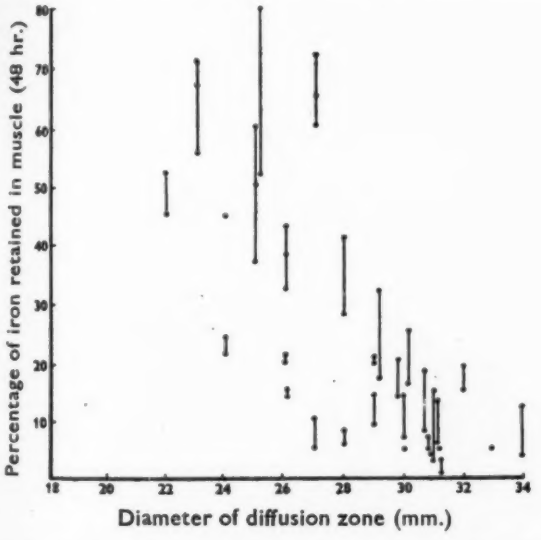


FIG. 3.—The relation between diffusion *in vitro* and muscle retention *in vivo* observed in various preparations of iron-dextran complex. *In vitro* measurement of the extent of diffusion of each complex in serum-agar was made after 6 days at 37° C.; *in vivo* measurement of the proportion of iron retained in rabbit muscles was made 48 hr. after injection. The vertical lines join points representing an individual preparation.

Histological Changes Following Injection of the Complex IDA

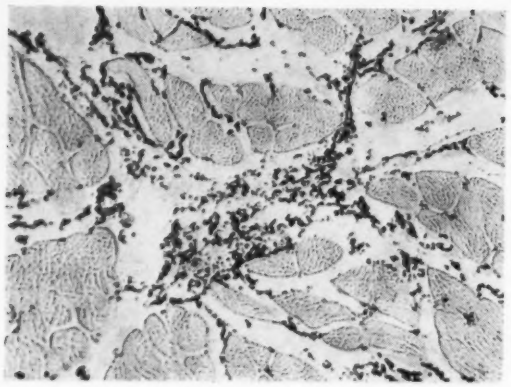
One Hour After the Injection.—There was a linear focus of damaged muscle along the line of the needle track, the damaged muscle fibres being swollen, eosinophilic, and often vacuolated. The mass of injection was lying free in the stroma between the muscle bundles and in the larger septa (Fig. 4*a*) ; some, however, had already entered the lymphatics and had been fixed in the littoral cells of the popliteal lymph nodes (Fig. 4*b*).

Eight Hours After the Injection.—A small focal area of muscle about 2 to 3 mm. across, presumably the precise locus of the injection, showed acute muscle damage. There were swollen eosinophilic fibres, sometimes hyalinized, sometimes vacuolated. Occasionally the sarcolemmal sheath appeared to have ruptured and amongst the fibres there was a mild infiltration of polymorphs, some being inside the sarcolemmal sheath (Fig. 4*c* and *d*). Practically no iron was detected by the Prussian blue reaction in the area of damaged muscle, and only small amounts were found where it had seeped away from the injection site along the septa. Here it produced no tissue reaction, but a little had already been taken up in the histiocytes.

Twenty-four Hours After the Injection.—At the locus of injection the polymorphs were almost completely replaced by macrophages (Fig. 4*e*), and some of the damaged muscle fibres showed a granular basophilia. No iron could be stained in the injection site at this time, either free or within macrophages, and the iron in the surrounding septal tissues was now partly taken up in the tissue histiocytes (Fig. 4*f*).

Forty-eight Hours After the Injection.—Macrophage infiltration of the focus of damaged muscle was intense, the macrophages being found both inside and outside the sarcolemmal sheaths, which were usually collapsed (Fig. 5*a*). Proliferation of sarcolemmal nuclei could also be recognized. The iron in the surrounding stroma was now almost entirely intracellular.

One Week After the Injection.—The inflammatory reaction had almost disappeared and a



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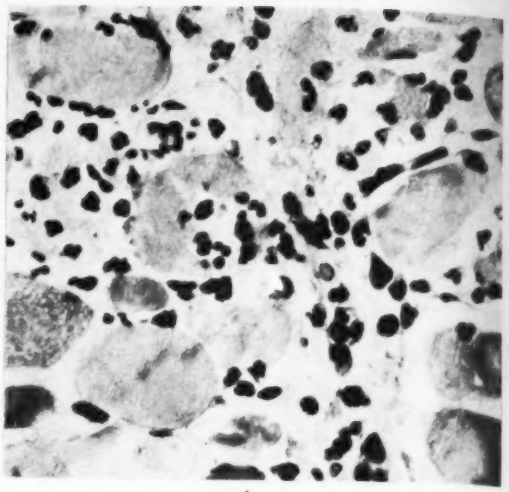

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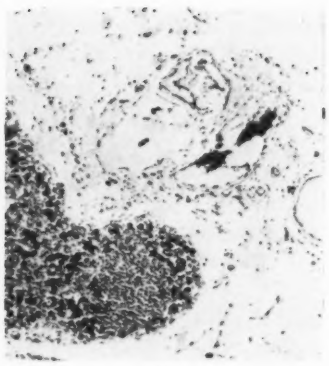

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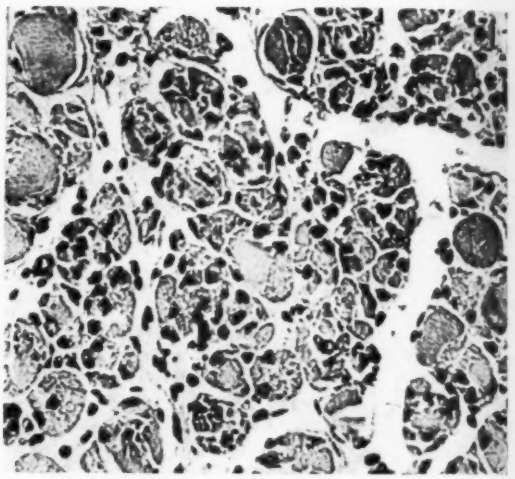

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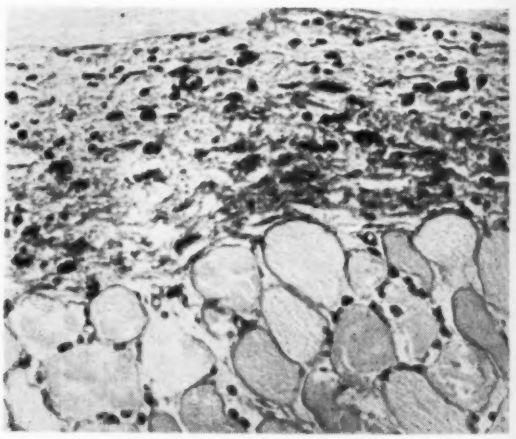

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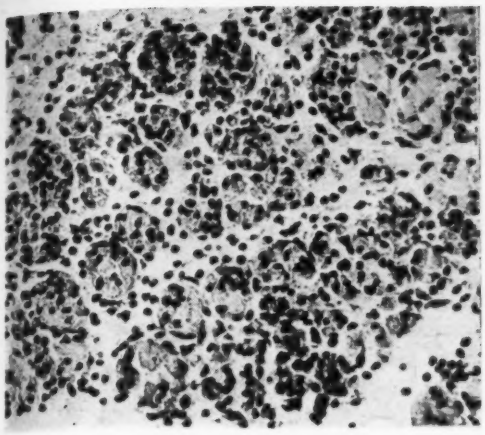
FIG. 4.—(*a*) One hr. after the injection of IDA. Prussian blue ×120. Intact muscle fibres with the injection material free in the stroma. (*b*) One hr. after the injection of IDA. Prussian blue ×42. Iron is seen in the lymphatics and in the lymph gland. (*c*) 8 hr. after injection of IDA. H. and E. ×22. The focus of degeneration and inflammation at the injection site. (*d*) 8 hr. after the injection of IDA. H. and E. ×480. Swollen, necrotic muscle fibres at the injection site with polymorphs in the stroma and inside the sarcolemmal sheaths. (*e*) 24 hr. after the injection of IDA. H. and E. ×250. The macrophage response at the injection site. (*f*) 24 hr. after the injection of IDA. Prussian blue ×200. The injection material in the septa away from the injection site is partly intracellular and partly extracellular.



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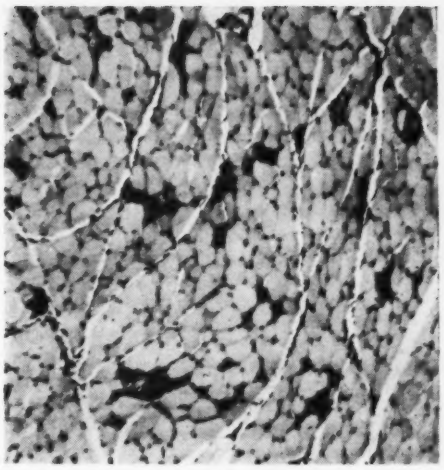

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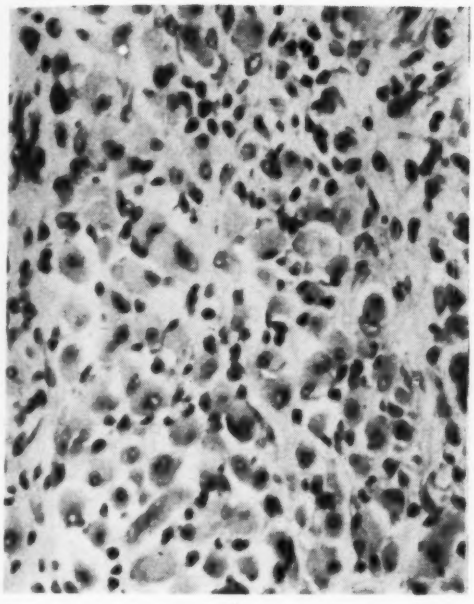

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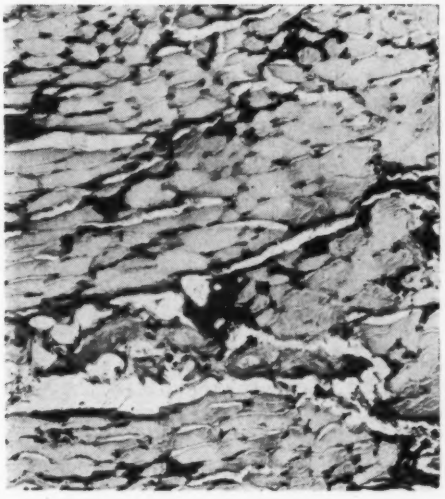

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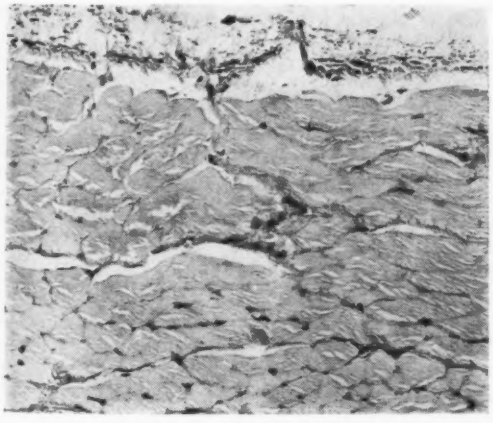

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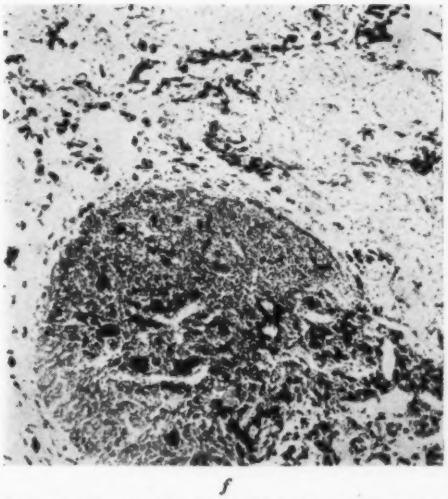

f

FIG. 5.—(*a*) 48 hr. after the injection of IDA. H. and E. ×180. Collapsed muscle fibres with proliferation of sarcolemmal nuclei and intense macrophage infiltration. (*b*) One week after the injection of IDA. H. and E. ×240. Regenerating muscle fibres at the injection site. (*c*) One month after the injection of IDA. Prussian blue ×80. Residual injection material in macrophages in the stroma. (*d*) 48 hr. after the injection of IDB. H. and E. ×65. The macrophage response at the many small scattered foci of muscle damage. (*e*) One month after the injection of IDB. Prussian blue ×80. Very heavy residual injection material in macrophages in the stroma. (*f*) Three months after the injection of IDB. Prussian blue ×75. Much residual injection material in regional lymph nodes and in the stroma.

process of active regeneration of muscle was seen at the injection site. The regenerating fibres were small, with basophilic cytoplasm and central nuclei (Fig. 5*b*). Prussian-blue-stained sections showed small amounts of iron in stromal histiocytes outside the zone of regeneration.

One Month After the Injection.—The area of muscle damage could only be recognized by finding an occasional small muscle fibre with a central nucleus. There was no residual cellular reaction or fibrosis. Iron could still be detected in histiocytes in the septa (Fig. 5*c*) in amounts little less than those seen at one week.

Three Months After the Injection.—The muscle now appeared entirely normal. Small amounts of iron were stainable, about as much as was seen at the end of one month.

Histological Changes Following the Injection of IDB, IPH, and IPV

All three iron preparations produced an inflammatory reaction at the site of injection, the pattern being identical with that described for IDA. The amount of necrosis, however, varied. The two iron polysaccharides IPH and IPV produced areas of necrosis equal or greater in extent to that described above, but the iron-dextran IDB showed a different appearance. No single large focus of damage developed; instead scattered foci of muscle damage involving groups of only two or three muscle fibres were seen (Fig. 5*d*). In all three cases regeneration of the damaged muscle was complete within one month.

Large and important differences were seen between the four preparations when sections stained for iron were examined. These differences are summarized in Table II. The iron-dextran IDB was less completely absorbed from the foci of muscle damage and was more quickly taken up by the tissue macrophages than was the product IDA. The iron polysaccharide IPV was as rapidly and completely removed from the injection site as was IDA, but there was a much more rapid take-up by the tissue macrophages of the iron which had diffused away from the injection site, so that very little free iron was seen after 24 hr. The iron polysaccharide IPH, whilst producing large areas of damaged muscle, showed considerable residual injection material in these damaged areas even after 48 hr. Little of it had been taken up by the tissue macrophages, and much of it appeared still to be extracellular after 48 hr. Prussian-blue-stained sections one and three months after the injection showed apparently undiminished numbers of iron-laden macrophages in the stroma amongst the muscle bundles (Fig. 5*e* and *f*).

Controls.—At the sites of injection of both dextran and saline no lesions were seen apart from those attributable to needle trauma, but occasionally a mild polymorph infiltration was observed around the larger particles of Indian ink.

Discussion

Histological Features

At the sites of intramuscular injection of the iron polysaccharides studied, an acute inflammatory reaction developed with local degenerative changes in the muscles. Regeneration rapidly occurred; repair was complete in less than one month, and left no residual damage in muscle, nerves, or other near-by tissues. Similar changes in injected muscles have been seen to a much more striking degree with other drugs. Lüthy (1955) and von Hochstetter (1955) have quoted Shallock's observations on the histological changes in the gluteal muscles of rabbits injected with a variety of drugs, including camphor, bismuth, liver extract, depot penicillin, and "Irgapyrin." In all

TABLE II

HISTOLOGICAL ASSESSMENT OF IRON DISTRIBUTION IN RAT MUSCLES AT VARIOUS TIMES AFTER INJECTION

Time After Injection	Iron Complex											
	IDA			IDB			IPV			IPH		
	Injection Site	Surrounding Stroma		Injection Site	Surrounding Stroma		Injection Site	Surrounding Stroma		Injection Site	Surrounding Stroma	
		Extracellular	Intracellular		Extracellular	Intracellular		Extracellular	Intracellular		Extracellular	Intracellular
8 hr. ..	±	++++	±	+++	++++	+	±	+++	++	+++	++++	+
24 „ ..	0	+++	±	+++	++	+	0	±	+++	+++	+++	++
48 „ ..	0	±	+	++	±	++	0	0	+++	++	++	+++
1 week ..	0	0	+	+	0	++	0	0	+++	++	0	++++
1 month	0	0	+	+	0	++	0	0	++	++	0	++++
3 months	0	0	+	+	0	++	0	0	+++	++	0	++++

0=No iron; ±, +, ++, +++, and ++++ indicate increasing tissue response.

cases, haemorrhages, thromboses, necrosis, and disintegration of muscle fibres occurred at the injection site, and were followed by the development of granulation tissue and subsequent formation of a fibrous scar. Glückert and Benoit (1952) reported similar damage following the injection of sulphonamide into the thigh muscles of guinea-pigs near the sciatic nerve. Considerable damage was also caused to the nerve.

The quantitative experiments showed great differences between the four iron preparations in the rate of their absorption from the injection site, but in all cases absorption was greatest during the first 72 hr., and little more occurred subsequently. These results were probably largely attributable to the changes developing in the muscle at the injection site, which suggested that active intervention by the tissue itself was essential for the absorption of iron-polysaccharide complexes deposited within it.

In their extensive study of the pharmacology of parenteral iron preparations, Brownlee, Bainbridge, and Thorp (1942) put forward the view that, "while necrosis is commonly seen with some compounds, this is regarded as indicating an unsuitable compound which is badly absorbed and therefore likely to give a poor haemoglobin response." No evidence in support of these statements was provided, nor was the nature of the "necrosis" to which they referred made clear. Nevertheless, the type of compound tested by these authors differed significantly from the iron-polysaccharide complexes to make it likely that their absorption mechanisms were quite distinct.

Relation to Absorption

Everett, Garrett, and Simmons (1954) showed that ionic iron was absorbed from a subcutaneous site primarily into the blood vessels, whereas a subcutaneous injection of plasma-bound iron passed almost exclusively into the lymphatics. The molecular size of the iron complexes studied here made it likely that absorption would take place by a lymphatic route. In fact, uptake into the lymphatics was rapid, stainable iron being found in the regional lymph nodes within an hour of injection. Clinical studies using Fe^{59}-labelled iron-dextran showed curves of absorption from the muscle similar to those given in Fig. 2, corresponding to IDA. These studies revealed, as might be expected, a considerable amount of individual variation. There is a suggestion from this and other work on intramuscular absorption that absorption is delayed if lymphatic drainage is retarded by immobilization of the injected limb (Grimes and Hutt, personal communication).

In considering the mechanism of absorption of the iron polysaccharide complexes a distinction must be made between the concentrated mass of material deposited at the injection site and the portion which diffuses into the surrounding tissues and is diluted by the extracellular fluid. Lymphatic absorption is dependent upon the rate of lymph flow and upon lymphatic capillary permeability; both of these are increased in an acute inflammatory reaction. The inflammatory focus at the injection site of the iron polysaccharide complexes might therefore be expected to enhance their absorption. Indeed, the central masses of the complexes IDA and IPV which appear histologically to be the best absorbed produced a much brisker inflammatory reaction than that which developed around the poorly absorbed complex IDB. Paradoxically, the central mass of the complex IPH was poorly absorbed, despite a very extensive inflammatory reaction. It can only be surmised that its molecular weight lay beyond the range capable of penetrating easily into the lymphatics: it also had a higher viscosity than the other polysaccharides, and this fact, together with other unknown physico-chemical properties, may have adversely affected its rate of absorption.

The macrophage also has an effect upon the rate of absorption of the iron complexes. Uptake by local macrophages renders the iron no longer immediately available to the body. In the present experiments, tissue macrophages accumulated abundantly in and around the injection site within 24 hr. In the central concentration of injected material the local inflammatory reaction was usually sufficient to remove the bulk of the injection before the macrophages appear. That portion of the injection which had diffused more widely was not removed before the arrival of the macrophages; its rapid uptake by these macrophages, as occurs with the complex IPV, must seriously limit its general availability to the body. This probably accounts for the lower absorption figures for IPV recorded in the quantitative experiment. The persistence of iron-laden macrophages in undiminished number in the stroma up to three months after the injection suggests that this iron may be permanently fixed. Thus the concept of an effective intramuscular depot, a familiar feature of many well-known drugs, is not applicable to the iron-polysaccharide complexes. Prompt absorption from the muscle or prolonged retention within it appear to be the only alternatives.

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References

Barnes, J. M., and Trueta, J. (1941). *Lancet*, **1**, 623.

Brownlee, G., Bainbridge, H. W., and Thorp, R. H. (1942). *J. Pharm., Lond.*, **15**, 148.

Drinker, C. K., and Field, M. E. (1933). *Lymphatics, Lymph and Tissue Fluid.* London: Baillière, Tindall and Cox.

Everett, N. B., Garrett, W. E., and Simmons, B. S. (1954). *Amer. J. Physiol.*, **178**, 45.

Glückert, H., and Benoit, W. (1952). *Nervenarzt*, **23**, 17

Hochstetter, A. von (1955). *Schweiz. med. Wschr.*, **85**, 1138.

Lüthy, F. (1955). Ibid., **85**, 1092.

Martin, L. E., Bates, C. M., Beresford, C. R., Donaldson, J. D., McDonald, F. F., Dunlop, D., Sheard, P., London, E., and Twigg, G. D. (1955). *Brit. J. Pharmacol.*, **10**, 375.

Middleton, G., and Stuckey, R. E. (1954). *Analyst*, **79**, 138.

Mowry, R. W., and Millican, R. C. (1953). *Amer. J. Path.*, **29**, 523.

Ventura, S., and Klopper, A. (1951). *J. Obstet. Gynaec. Brit. Emp.*, **38**, 173.

Brit. J. Pharmacol. (1957), **12**, 115.

INHIBITION BY MORPHINE OF THE RELEASE OF ACETYLCHOLINE FROM THE INTESTINE OF THE GUINEA-PIG

BY

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In experiments on the isolated small intestine of the guinea-pig, morphine inhibited the release of acetylcholine (ACh) into the bath fluid. Experimental evidence is presented which suggests that the reduced release of ACh could not be explained by an inhibition of the synthesis of ACh, nor by stabilization of the bound form of ACh in the tissue. Apparently morphine reduces the excitability of postganglionic structures and thereby the liberation of ACh from nerve endings during the process of excitation.

The specific paralysing effect of morphine on the guinea-pig intestine (Schaumann, 1955) can probably be explained by an inhibition of the release of acetylcholine (ACh) (Paton, 1956 ; Schaumann, 1956b). The present experiments were carried out to determine the mechanism by which morphine prevents the liberation of ACh. Three possibilities were considered: (1) that there is an inhibition of the process of excitation which physiologically provokes the liberation of ACh ; (2) that there is a stabilization of ACh in the tissue in its bound form ; and (3) that there is an inhibition of the synthesis of ACh.

METHODS

Guinea-pigs were killed by a blow on the neck and bled. The small intestine was taken out, emptied of its contents, and divided into 12 to 16 equal pieces. Two pieces from different parts of the gut were combined into one sample to reduce the effect of the variations in choline acetylase content along the length of the intestine (Feldberg and Lin, 1950). The samples were incubated at 36 to 37° C. in 10 ml. Mg-free Tyrode solution in the presence of eserine salicylate 10^{-5} (w/v) unless stated otherwise, the pieces being tied off at both ends to prevent the mucus in the lumen from oozing into the bath fluid. The bath was aerated with a mixture of 95% O_2 and 5% CO_2. After incubation the bath fluid was diluted with half its volume N/100 HCl and assayed for ACh on the eserinized frog rectus abdominis muscle. In these experiments, standard dilutions of ACh were made up in bicarbonate-free frog Ringer solution. ACh was extracted from the tissue by the procedure of Chang and Gaddum (1933). When the ACh content of the tissue or the ACh liberated from ground tissue was determined, part of the extract was briefly boiled with NaOH to destroy the ACh, and then neutralized. Equivalent amounts of inactivated extract were added to the reference solutions of ACh. Morphine was added to the controls before assay to allow for a possible sensitization of the frog rectus to ACh by morphine (Torda and Wolff, 1947). ACh is expressed in terms of ACh chloride ; the concentrations of morphine refer to those of morphine hydrochloride.

RESULTS

Potency of Morphine in Inhibiting the Release of ACh.—As in previous experiments on distended strips of intestine (Schaumann, 1956b), morphine was found to reduce the liberation of ACh from undistended intestinal tissue. Pieces of intestine were incubated in the presence of different concentrations of morphine and the amounts of ACh released into the bath fluid determined ; the results are summarized in Table I. A concentration as low as 10^{-7} produced a significant reduction in the ACh output. However, the effect increased only slightly with the dose, and even a concentration of morphine of 10^{-4} did not abolish the release altogether.

TABLE I

INHIBITION OF THE RELEASE OF ACh (μG./G./HR.) BY INCREASING CONCENTRATIONS OF MORPHINE

Expt. No.	Control*	Morphine			
		10^{-7}	10^{-6}	10^{-5}	10^{-4}
1	3·85	2·5	2·7	1·8	1·8
2	3·5	3·0	2·6	2·9	2·4
3	3·65	3·2	2·4	1·4	1·3
4	5·2	2·4	3·3	2·0	1·7
5	3·5	1·8	2·0	2·1	1·7
Mean	3·9	2·6	2·6	2·0	1·8

* Mean from two samples.

In previous experiments (Schaumann, 1955) in which morphine was allowed to act on the intestine for a long time, it was found that the preparatory phase of the peristaltic reflex was abolished only for a short time after the morphine had been given, and that, in spite of the continued presence of morphine, this reflex returned in the course of 30 min. It is thus possible that morphine produced a complete inhibition of the ACh output at the beginning of the experiment which wore off during the one hour of incubation. The results obtained by determining the total amount of ACh released during incubation are consistent not only with this assumption but also with the possibility that morphine produced a continuous, incomplete inhibition. Therefore the release of ACh was determined throughout the experiment at intervals of 7.5 min. Such an experiment is illustrated in Fig. 1. In the absence of morphine there was a rapid increase in the liberation of ACh in both samples after the addition of eserine. After about

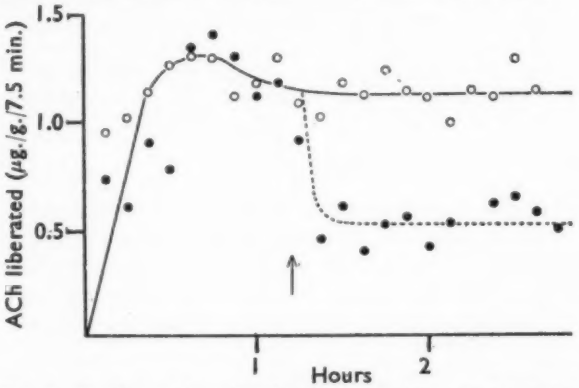

FIG. 1.—Continuous determination of ACh output. Two samples were incubated in 6.5 ml. Tyrode solution each; 5 ml. was exchanged every 7.5 min. and assayed for ACh. At arrow, morphine 10^{-7} was added to one sample (solid circles) and kept in the bath to the end of the experiment.

90 min. the rate of release of ACh reached a value at which, in the control samples, it remained constant to the end of the experiment. When morphine 10^{-7} was added the output of ACh fell by more than half, and did not rise again during the following 90 min. High concentrations of morphine were also used, but an initial complete inhibition followed by recovery, or even a gradual decrease of the inhibitory effect in the course of time, was never observed.

In these experiments with repeated exchanging of the bath fluid, greater yields of ACh/g./hr. were obtained than in those where there was one hour's incubation without renewal of the bath solution. This is probably due to an equilibrium between free and bound ACh as postulated by Mann, Tennenbaum and Quastel (1939) and by Brodkin and Elliot (1953). The ACh accumulating in the bath probably prevented its further release from the tissue.

Liberation of ACh from Ground Intestinal Tissue.—ACh is present in the tissue in a bound form from which it is split off by reactions as yet unknown. Morphine might reduce the release of ACh by inhibiting these reactions and thereby stabilizing its inactive precursor in the tissue. In that case, morphine should be expected to reduce

TABLE II

RELEASE OF ACh FROM GROUND TISSUE IN THE ABSENCE AND IN THE PRESENCE OF MORPHINE

Expt. No.	Tissue ACh μg./g.			ACh Liberated μg./g.	
	After Grinding	After 1 hr. Incubation			
		Control	Morphine 10^{-4}	Control	Morphine 10^{-4}
	1	2	3	4	5
1	2·3	0·5	0·5	2·1	2·2
2	6·6	1·7	1·0	4·0	4·3
3	5·7	1·0	0·9	8·3	9·7
4	6·2	1·7	1·2	7·6	8·1
5	8·1	1·1	1·0	4·7	4·4

the release from ground tissue also. The following procedure was adopted to test this possibility. The intestine of one guinea-pig was divided into six samples which were ground with quartz sand in ice-cold mortars. After this grinding, the ACh content of two samples was assayed ; the mean of the determination is given in col. 1 of Table II. The other four samples were incubated for one hour, two as controls and two in the presence of morphine 10^{-4}. They were then centrifuged at 3,000 rev./min. for 5 min. and the ACh was determined in the residue and in the supernatant fluid. Cols. 2 and 3 show that little ACh was left in the residue, most of it being liberated from the tissue during incubation. In most experiments the cell debris of the samples to which morphine had been added contained a little less ACh than the controls, and in 4 out of 5 experiments a little more free ACh was found in the supernatant in the presence of morphine (cols. 4 and 5). Apparently, these high concentrations of morphine enhanced rather than depressed the release of ACh from its bound form.

Effect of Morphine on the Synthesis of ACh.—The possibility must be considered that the reduced rate of release of ACh into the bath fluid might also result from inhibition of the synthesis of ACh. It is usually assumed that ACh is first synthesized in the tissue in a bound form and then

released from this inactive precursor (Vartiainen, 1934; Trethewie, 1938). This is supported by the observation that there was an increase in the ACh content of the tissue during incubation of intact pieces of intestine without eserine. This increase could not be due to primary synthesis and subsequent binding of free ACh, since free ACh would at once have been destroyed by the cholinesterase. Experiments in which such an increase was obtained are illustrated in Table III.

TABLE III
INCREASE OF ACh CONTENT OF THE TISSUE DURING INCUBATION FOR ONE HOUR WITHOUT ESERINE

Expt. No.	ACh Content in μg./g.	
	Before Incubation	After Incubation
1	8·7	9·8
2	8·2	9·1
3	5·8	7·95
4	6·3	8·4

During incubation with eserine, there was a much greater increase in the ACh content of the tissue. In the experiments summarized in Table IV, strips of intestine were incubated for one hour in the presence of eserine either with or without addition of morphine 10^{-5}. Each value is the mean of two samples. Inhibition of the ACh synthesis by morphine should have resulted in a diminished

TABLE IV
INCREASE OF ACh CONTENT OF THE TISSUE DURING INCUBATION IN THE ABSENCE AND IN THE PRESENCE OF MORPHINE 10^{-5}

Expt. No.	ACh μg./g.				
	Before Incubation	After Incubation		Increase During Incubation	
		Control	Morphine	Control	Morphine
1	7·6	11·4	11·4	3·8	3·8
2	7·6	10·3	11·6	2·7	4·0
3	9·9	17·5	18·7	7·6	8·8
4	10·3	15·1	15·8	4·8	5·5
5	9·1	10·4	11·6	1·3	2·5

increase in ACh content. On the contrary, the last two columns of Table IV show that morphine slightly enhanced the increase in ACh content of the tissue. The difference was small and can be explained by the reduced release of ACh from its bound form. Apparently, morphine does not inhibit the synthesis of ACh.

DISCUSSION

The low concentrations of morphine which abolished the peristaltic reflex and nicotine contractions proved sufficient to reduce the liberation of ACh. This confirms the result of Paton (1956) and suggests that the effects of morphine on the guinea-pig intestine are due to inhibition of the release of ACh. The following facts suggest that this ACh is derived from nervous elements: (1) there is no correlation between muscular activity and ACh output—for example, atropine abolished both the peristaltic reflex and the nicotine contractions as did morphine (Schaumann, 1955), but not the release of ACh from the intestine (Schaumann, 1956b); (2) morphine abolished the contractions of the guinea-pig's duodenum to vagal stimulation by paralysing postganglionic structures (Schaumann, 1956a). This can also be accounted for by an inhibition of the release of ACh, which in this case certainly is of nervous origin.

Although morphine was effective in concentrations as low as 10^{-7}, some ACh was released even in the presence of concentrations of morphine 1,000 times higher. This ACh may have originated from non-nervous structures, for ACh is probably liberated not only from nervous elements, but also from muscle cells and the mucosa (Feldberg and Lin, 1950). The release of ACh from these structures may not be affected by morphine.

The effectiveness of morphine seems to be limited to the intact cell, for it did not prevent the release of ACh from ground tissue. Thus the results of Torda and Wolff (1947) and of Kumagai, Ebashi and Takeda (1954), who found that morphine had no significant effect on the formation of ACh, are inconclusive, because they were obtained from experiments on cell-free extracts. However, similar results were obtained in the present experiments on intact intestinal tissue. The evidence thus indicates that morphine produces little if any inhibition of the synthesis of ACh.

Apparently, morphine inhibits the excitatory processes which release ACh from nerve endings. According to Schaumann, Giovannini and Jochum (1952), the paralysing activity of morphine-like analgesics runs parallel with their analgesic activity. This suggests that the action of morphine and its substitutes on the central nervous system may likewise be due to inhibition of the release of ACh at cholinergic synapses.

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References

Brodkin, E., and Elliot, K. A. C. (1953). *Amer. J. Physiol.*, **173**, 437.

Chang, H. C., and Gaddum, J. H. (1933). *J. Physiol.*, **79**, 255.

Feldberg, W., and Lin, R. C. Y. (1950). Ibid., **111**, 96.

Kumagai, H., Ebashi, S., and Takeda, F. (1954). *Jap. J. Pharmacol.*, **4**, 24.

Mann, P. J. G., Tennenbaum, M., and Quastel, J. H. (1939). *Biochem. J.*, **33**, 822.

Paton, W. D. M. (1956). *Abstr. 20th int. physiol. Congr.*, Bruxelles, 708.

Schaumann, O., Giovannini, M., and Jochum, K. (1952). *Arch. exp. Path. Pharmak.*, **215**, 460.

Schaumann, W. (1955). *Brit. J. Pharmacol.*, **10**, 456.

—— (1956a). *Arch. exp. Path. Pharmak.*, **229**, 432.

—— (1956b). *Nature, Lond.*, **178**, 1121.

Torda, C., and Wolff, H. G. (1947). *Amer. J. Physiol.*, **151**, 345.

Trethewie, E. R. (1938). *Aust. J. exp. Biol. med. Sci.*, **16**, 225.

Vartiainen, A. (1934). *J. Physiol.*, **82**, 282.

Brit. J. Pharmacol. (1957), **11**, 119.

THE ACTION OF MORPHINE AND RELATED SUBSTANCES ON CONTRACTION AND ON ACETYLCHOLINE OUTPUT OF COAXIALLY STIMULATED GUINEA-PIG ILEUM

BY

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Morphine depresses the twitch and tetanus of stimulated guinea-pig ileum by reducing acetylcholine released from cholinergic nerve endings. Acetylcholine output per shock falls to roughly the same residual amount at varying stimulation rates. Since normal output per shock declines with increasing stimulus frequency, the proportionate effect of morphine diminishes as stimulus frequency rises. Acute "tolerance" to morphine and a state of "morphine-dependence" can be produced. Phenadoxone, dihydromorphinone, metopon, methadone, and heroin are more active, codeine and pethidine less active, than morphine. Nalorphine also depresses the twitch and can desensitize the gut both to itself and to morphine.

Despite the widespread use of morphine there is still no agreement as to the mode of action, either when used as an analgesic or for depressing the activity of the intestine. It has been suggested that its anticholinesterase activity causes the central actions; but it is a relatively feeble inhibitor of cholinesterase, and much more active inhibitors do not reproduce its effects. On the intestine, attention has focused on morphine's ability either to depress propulsion, or to increase the tone of the gut. An investigation of the action of opiates on intestinal loops in the dog by Vaughan Williams and Streeten (1950, 1951) has clarified some apparent contradictions in previous experimental results, and shown how the *stimulant* action of morphine could lead to a failure of transport of intestinal contents. Recent work by the Schaumanns (Schaumann, Giovannini and Jochum, 1952; Schaumann, 1955) and by Kosterlitz and Robinson (1955) has indicated, however, that the action of morphine may be through a *depression* of nervous mechanisms involved in peristalsis.

The method recently developed for exciting the nervous tissue in the wall of the small intestine (Paton, 1955) offered a suitable means for analysing the activity of morphine further. If electrodes are placed within and without the lumen of a strip of guinea-pig ileum immersed in Krebs solution and current is passed between them, vigorous twitch-like contractions can be obtained to single shocks, which are due to excitation of postganglionic cholinergic nerves. The contractions are reproducible, are antagonized by small doses of atropine, are potentiated by anticholinesterases, and resist ganglion-blocking agents. Further, it has proved possible to measure the output of acetylcholine in response to the excitation of nervous tissue in the wall of the gut.

In this paper the effect of morphine (and related drugs) on the twitch of the intestine and on its acetylcholine output is described.

A short account of some of the results obtained with morphine was given elsewhere (Paton, 1956).

METHODS

The experiments were all made on strips of guinea-pig ileum suspended in Krebs solution, bubbled with 95% O_2 and 5% CO_2. Rectangular current pulses usually of 1 msec. duration, and of sufficient strength to produce a maximal response to a single shock, were applied to the electrodes; the intraluminal electrode was made the anode. When necessary, repetitive stimulation was used to produce a partially or completely fused tetanus for a period of a few seconds or a minute. The contractions of the gut were recorded either on a smoked drum or by a light ink-recorder. The bath was kept at 35 to 37° C. The capacity of the bath in which the gut and electrodes were immersed was normally 50 ml.

The assay of acetylcholine output was conducted on another strip of guinea-pig ileum treated with 5 μg./l. of neostigmine and 10 mg./l. of morphine. As soon as the first observation was made that morphine reduced the output of acetylcholine by the gut,

the possibility suggested itself that morphine might prevent the violent intermittent spasms of the guinea-pig ileum which occur when it is exposed even to low concentrations of anticholinesterases, and greatly diminish its usefulness for assay. This proved to be the case, and the intestine sensitized with neostigmine (which appeared more suitable than eserine) and quietened with morphine furnished a very convenient assay method which was used in all subsequent experiments. (A paper describing the method is in preparation.) The experiment consisted of transferring to the assay bath (7.5 ml.) an aliquot of the fluid from the donor bath holding the strip of stimulated intestine. The donor bath fluid contained eserine 10^{-6}. Sensitivity of the recipient intestine down to 1 ng. of acetylcholine added to the bath was easily obtained, and assays could be repeated every 1½ to 2 min. if required.

The following drugs were employed: acetylcholine chloride; methadone hydrochloride; atropine sulphate; codeine sulphate; dihydromorphinone hydrochloride; eserine sulphate; heroin hydrochloride; histamine phosphate; hyoscine hydrobromide; morphine sulphate; metopon hydrochloride (methyldihydromorphinone hydrochloride); neostigmine methylsulphate; pethidine hydrochloride; phenadoxone hydrochloride. The doses are all given in terms of the salt, except histamine, which is given as base.

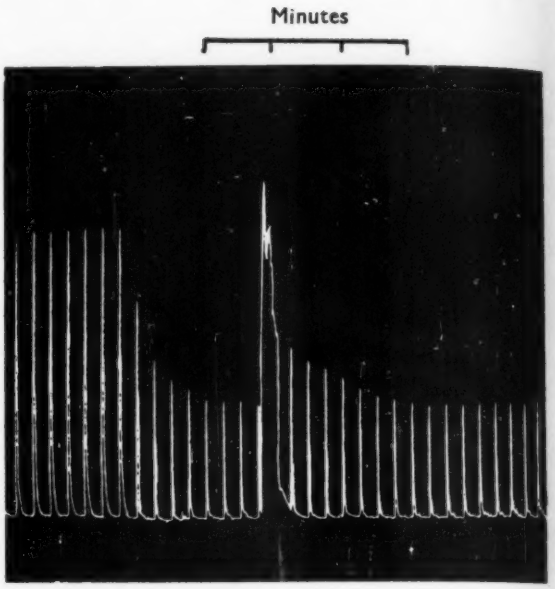

FIG. 1.—Effect of morphine 4 μg. on twitches of guinea-pig ileum stimulated by single maximal shocks coaxially every 15 sec., and transient partial recovery of twitch after a brief tetanus.

RESULTS

The Depression of the Twitch of the Guinea-pig Ileum by Morphine.—Morphine in low doses reduces the twitch of a guinea-pig ileum (Figs. 1, 2, 3, 5, 6, 7, 10, 11, 13). The effect is of relatively rapid onset and reaches a plateau within 1 or 2 min. If the morphine is left in the bath thereafter variable results occur. Sometimes a modest spontaneous recovery takes place (Figs. 2, 3, 10). At other times the block is maintained at its initial level up to 15 to 30 min. After a brief exposure of 1 to 3 min., a fairly rapid recovery takes place after washing out (Figs. 5, 7). But, if the morphine is left in for a substantial length of time, recovery is much slower (Figs. 2, 3); often, immediately after washing out, a temporary recovery is succeeded by a yet deeper depression of the twitch (Figs. 2, 10).

The dose-response curve of the response to morphine is hard to establish because of the development of tolerance as described later. But if doses are spaced 30 min. or an hour apart, and if exposures are only long enough to record the maximum effect, reproducible

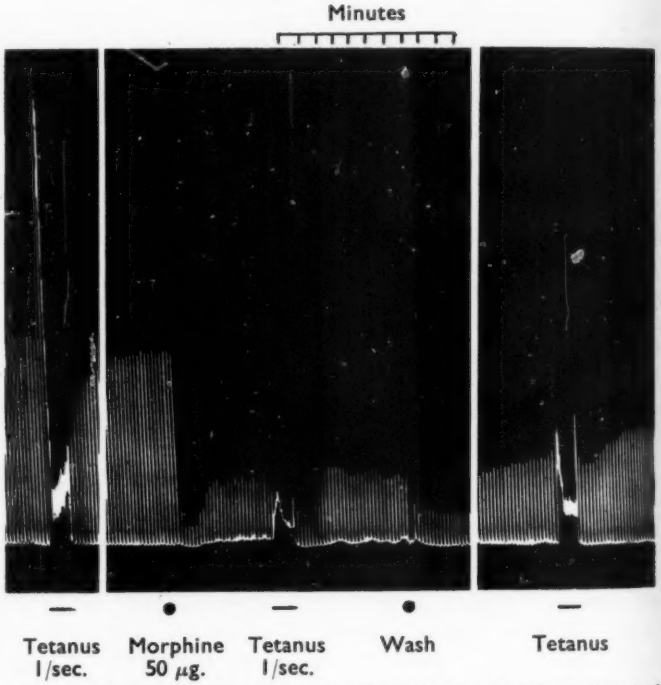

FIG. 2.—Effect of morphine 50 μg. on twitches (6/min.) and tetanus (1/sec.) of guinea-pig ileum, showing partial recovery of twitch in presence of morphine, characteristic effect of morphine on shape of tetanic response, further reduction of twitch on washing out morphine, and incomplete recovery 20 min. after washing out morphine.

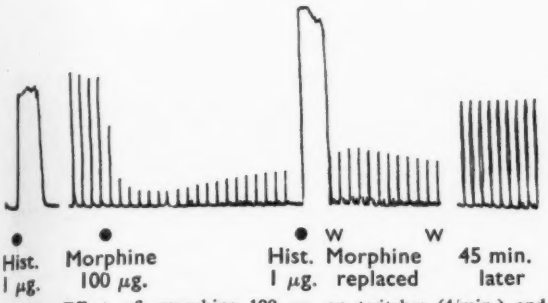

FIG. 3.—Effect of morphine 100 μg. on twitches (4/min.) and histamine response of guinea-pig ileum. Histamine 1 μg. was added to bath while stimulation was temporarily stopped. The twitch recovered slightly in the presence of morphine, the histamine response was increased, and the gut recovered only slowly from the relatively prolonged exposure to morphine.

responses to morphine can be obtained. Even then, however, the first response to morphine is often proportionately larger than later responses (Fig. 10). The threshold dose of morphine is about 10^{-8}; maximal action, in the absence of tolerance, is achieved by about 10^{-6} morphine.

Morphine also modifies the tetanic response of the gut to repetitive stimulation. The chief change is that its initial sharp spike is relatively more depressed, and the sustained part of the tetanic response less affected (Fig. 2). After the tetanus the twitch may be reduced (Fig. 2) or partially restored for a short time (Fig. 1).

The depression produced by morphine can be rapidly reversed by anticholinesterases such as eserine or neostigmine in concentrations of 10^{-7}.

The Effect of Drugs Related to Morphine.—All the drugs tested which possess an analgesic action similar to that of morphine also exerted a depressant effect on the twitch of guinea-pig ileum. Figs. 4, 5, 6, 7, 8 illustrate some typical results with the drugs used. The absolute comparison of potency between one drug and another is difficult to make quantitative, because tolerance develops with them all. But if those responses are selected which were either (1) the result of the first administration of an opiate, or (2) were obtained during a series of small well-spaced doses, or (3) were obtained over a period where tolerance was developing uniformly so that a correction could be made for it, approximate quantitative comparisons can be made which are fairly reproducible. Table I summarizes the estimates of relative potency so obtained. Fig. 9 gives three dose-response curves from three separate experiments,

The activity of a compound on the ileum is well related to its analgesic potency. Thus codeine was relatively ineffective, although with doses of 50 μg. to 1 mg. convincing effects could be produced. On the other hand, phenadoxone, methadone,

TABLE I

APPROXIMATE RELATIVE POTENCY OF MORPHINE-LIKE SUBSTANCES IN BLOCKING THE TWITCH OF THE GUINEA-PIG ILEUM

Phenadoxone	100
Dihydromorphinone	70
Metopon	55
Methadone	30
Heroin	30
Morphine	15
Codeine	0·7

dihydromorphinone, metopon, and heroin were all more active than morphine.

Pethidine presented a special problem, since it is known to have an atropinic action of its own which would depress the twitch independently of the type of activity here being analysed. But it was found that evidence for a morphine-like action could be obtained in the following way. It had been observed that after atropine the response to injected acetylcholine was reduced considerably more than was the twitch, so that the "acetylcholine-equivalent" of the twitch (the dose which produces a contraction of the same height as the twitch) may increase 5- to 10-fold. With pethidine, on the other hand, although the response to injected acetylcholine is reduced as the twitch height diminishes, the antagonism to acetylcholine is not great (Fig. 8) and the acetylcholine equivalent of the twitch in fact rises. The effect of pethidine, therefore, is perfectly compatible with that of a drug having a mild atropinic action and a distinct morphine-like effect. The total effect of pethidine on the gut is roughly half as great as that of morphine, so that its specific morphine-like activity must be lower still.

The drugs mentioned all resembled morphine in other ways. The depression by any of these compounds usually comes on fairly quickly, and recovery after washing out is slower with a prolonged exposure, or when a large effect was produced. With all of them tolerance was liable to develop (Fig. 4). The drugs do, however, differ in their time course of action. In general codeine tended to reach its peak action quickest, morphine was usually (but not always) somewhat slower and the other substances tested (especially phenadoxone) had a still slower development of action. Thus, in Fig. 4, phenadoxone exerts the most slowly developing effect; in Fig. 6, methadone acts more slowly than morphine, though present in more powerful dosage. There was, in short, an inverse relation between potency on the one hand, and speed of onset or offset of action on the other.

Tolerance.—It was mentioned above that the sensitivity of the preparation diminished with repeated large doses. Even with small doses repeated at intervals of about 15 min., sensitivity often declined. In any experiment in which

FIG. 4.—Effects of phenadoxone, metopon, and dihydromorphinone on twitches (6/min.) of guinea-pig ileum, showing development of tolerance (compare the two responses to phenadoxone 3 μg.) and varying rates of onset of action. The times indicated in this and subsequent figures give the interval between doses of opiate.

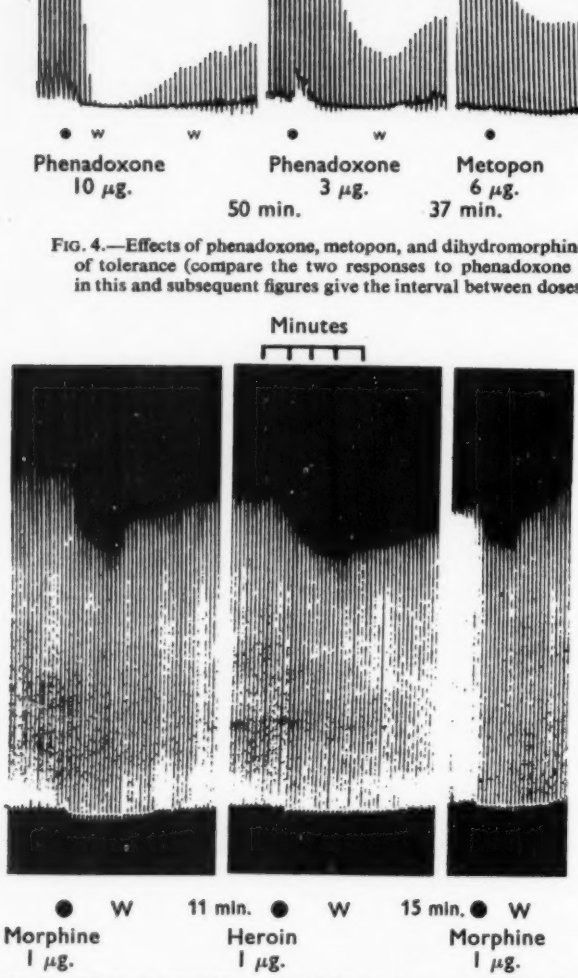

FIG. 5.—Effects of morphine 1 μg. and heroin 1 μg. on twitches of guinea-pig ileum (6/min.).

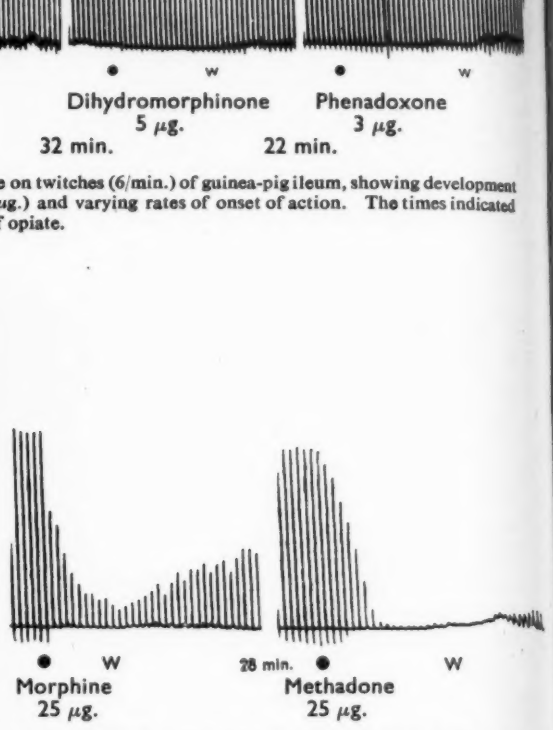

FIG. 6.—Effects of morphine 25 μg. and methadone 25 μg. on twitches of guinea-pig ileum (6/min.).

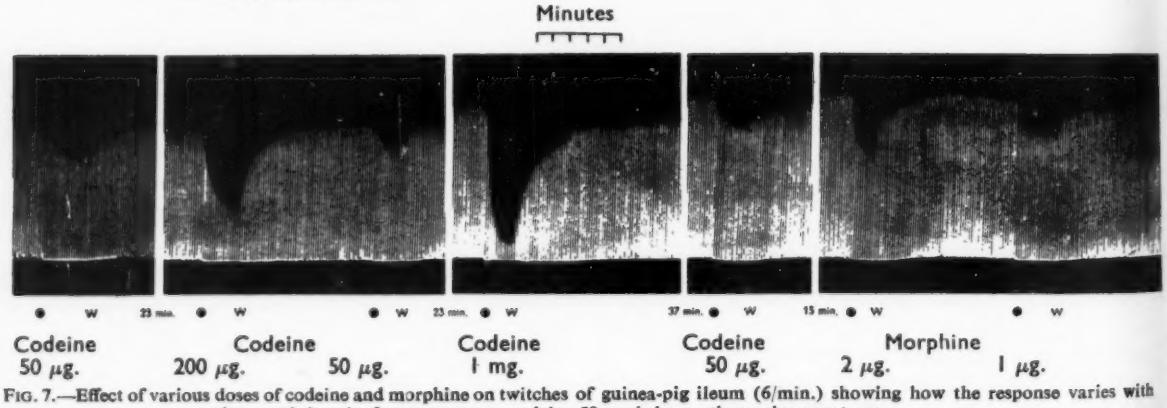

FIG. 7.—Effect of various doses of codeine and morphine on twitches of guinea-pig ileum (6/min.) showing how the response varies with dose, and that the first response to codeine 50 μg. is larger than subsequent ones.

repeated administrations occurred over a period of hours, the intestine gradually became less sensitive. Sometimes, but not always, recovery of the twitch could be demonstrated despite the continued presence of a dose of morphine; this recovery, however, was never complete (Figs. 2, 3, 10). Although in one experiment tetanic stimulation appeared to accelerate the development of tolerance, this could not be substantiated. In general, the tolerant intestine showed more spontaneous activity than normal, and this was sometimes specially marked on washing out a dose of opiate.

It proved difficult to control the sensitivity of the preparation satisfactorily, and it has not been possible hitherto to devise a reliable method either of avoiding tolerance completely, or of producing it to a known degree. But Fig. 10 illustrates one of the more reproducible phenomena. First of all a small dose of morphine (50 μg.) is given. After an interval for recovery (90 min.), the dose is repeated, producing a nearly comparable effect. Then, without removing this dose, the same dose is administered again; although this total dose is normally adequate to produce a complete block, now it produces no further effect. A much larger dose is likewise ineffective. The depression of the twitch seems to be fixed at a height determined by the effect of the first small dose chosen. If the morphine is now washed out the twitch height (sometimes after a transient recovery) is reduced still further and only slowly recovers. In order to restore the twitch height rapidly to its previous level, the simplest procedure at this stage is to add morphine; a total of 10 mg., sufficient to produce a short-lived contraction of the gut, has to be applied before the twitch height corresponds to its original level. Removing the morphine again leads to a reduction of the twitch. It is possible, in this way, to convert the preparation from a state in which a small dose of morphine depresses its contraction, to a state in which the intestine is dependent on the presence of morphine to maintain its normal response. The phenomenon of "tolerance" could not be produced with hyoscine or atropine. If these are given repeatedly their action on this preparation never decreases, and often increases slightly owing to the difficulty of washing the atropine out completely. Atropine continues to be normally active in gut which has been fully desensitized to morphine.

The Action of Nalorphine.—When the possibility of antagonism between nalorphine and morphine is tested, nalorphine proves to have itself a depressant effect on the intestine (Fig. 11).

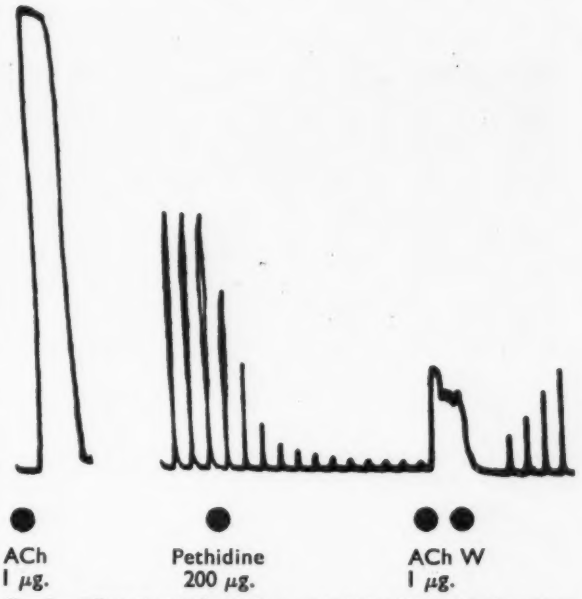

FIG. 8.—Effect of pethidine 200 μg. on twitches (4/min.) and response to acetylcholine 1 μg. by guinea-pig ileum.

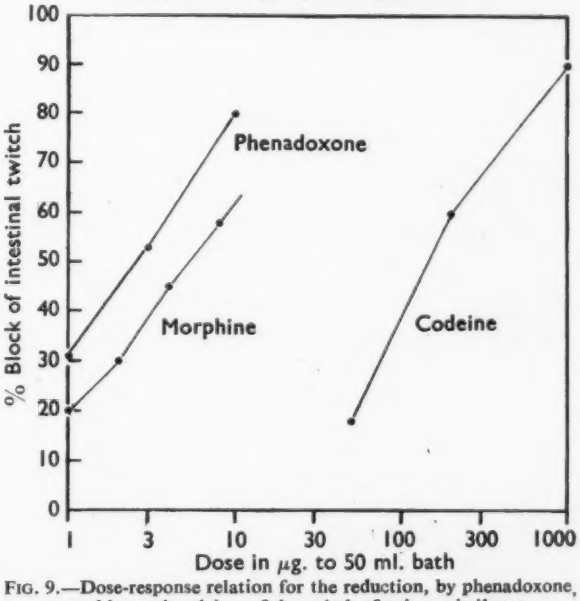

FIG. 9.—Dose-response relation for the reduction, by phenadoxone, morphine and codeine, of the twitch of guinea-pig ileum.

This is exerted in small doses and made the demonstration of an antagonism correspondingly complicated. It is possible, by giving a large dose of nalorphine and allowing the intestine to recover from it, to show that morphine had less effect thereafter. But the same effect is produced by a large dose of morphine. Further, acute tolerance to nalorphine can be produced in just the same way as to morphine (Fig. 12). The two drugs could not, in fact, be distinguished qualitatively, in

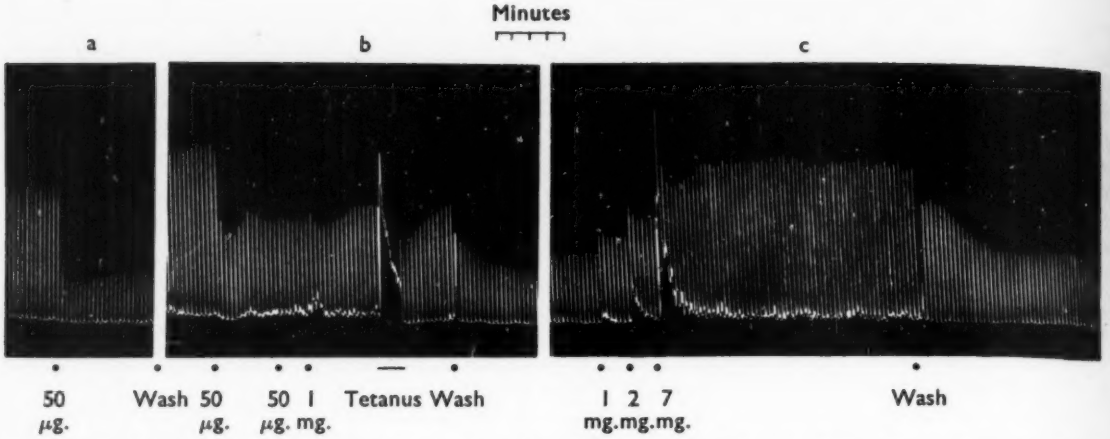

FIG. 10.—Development of "acute tolerance" and "morphine dependence" by guinea-pig ileum. Doses shown are of morphine. Twitches 4/min.; tetanus 5/sec. Between (*a*) and (*b*) 88 min.; between (*b*) and (*c*) 22 min. See text for other details.

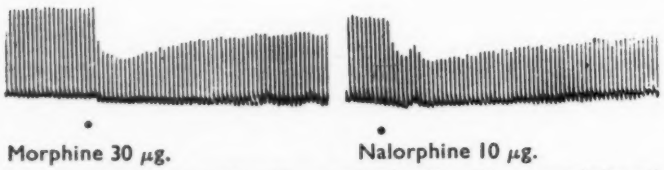

FIG. 11.—Effect of morphine 30 μg. and nalorphine 10 μg. on twitches of guinea-pig ileum (6/min.).

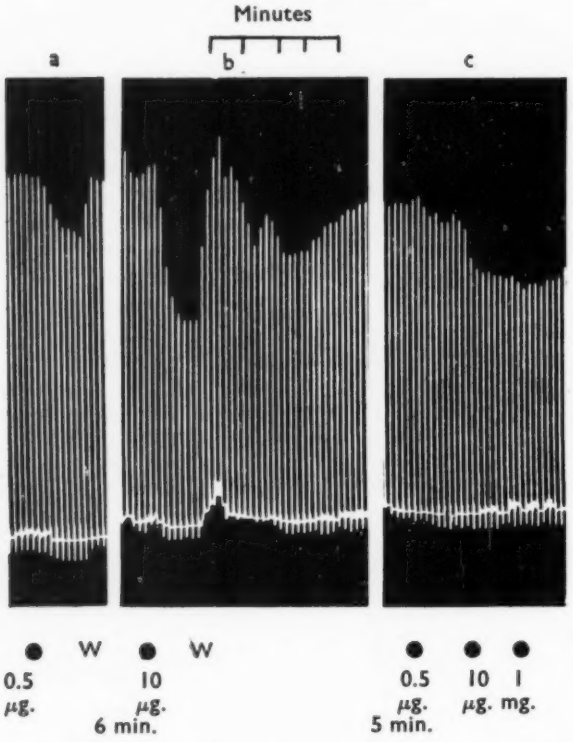

FIG. 12.—Development of "acute tolerance" to nalorphine. (*a*) Effect of 0.5 μg. nalorphine; (*b*) effect of 10 μg. nalorphine; (*c*) effect of 0.5 μg. nalorphine, followed by additions of 10 μg. and 1 mg. nalorphine. Twitches 6/min.

respect of their ability to depress the gut or to make it resistant to opiates. The results so far obtained suggest that on the intestine nalorphine should be regarded as an analogue rather than as a specific antagonist to morphine, although the experiments do not exclude that tolerance can be produced more rapidly with nalorphine.

The Analysis of the Action of Morphine.—The depressant action of morphine on the twitch of the intestine cannot be due to any local anaesthetic effect, such as cocaine displays. The threshold for maximal excitation was not changed by morphine and brief stimuli remained effective. When tested on frog sciatic nerve, stripped of its sheath, morphine in concentrations up to 10^{-2} failed to block nervous transmission (J. W. Thompson; personal communication).

If nervous tissue is normally responsive in the presence of morphine, then the reduction of the twitch height must be due to a reduction of acetylcholine output from the nerve endings, or to a competitive antagonism to the acetylcholine at the receptor groups, or a direct effect on the muscle itself. The first test to distinguish these possibilities was made by comparing the response to stimulation with the response to injected acetylcholine, in the presence of morphine. Fig. 13 shows a typical experiment. The twitch could at first be matched against a dose of 1.2 μg. of acetylcholine added to the bath. When morphine was given, the twitch was greatly reduced, but the response to acetylcholine was unaltered. Similar results were obtained with heroin and codeine. It was also found that sometimes the response to histamine was in fact augmented by morphine (Fig. 3).

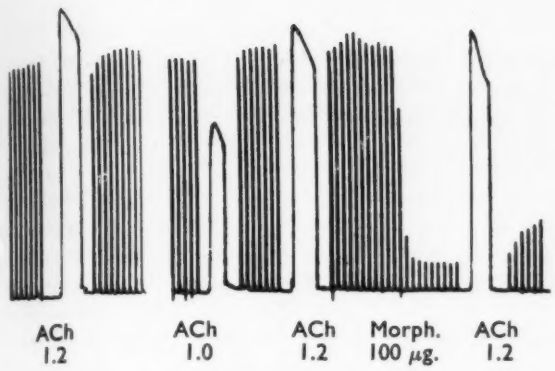

FIG. 13.—Comparison of the effect of morphine on the twitch of the guinea-pig ileum (6/min.) with its effect on the response to acetylcholine 1.2 μg. After each injection of acetylcholine the bath was washed out.

These results exclude the possibility that morphine either antagonizes acetylcholine after release or impairs in any way the contractile ability of the muscle. One must, therefore, suppose that morphine reduces the output of acetylcholine by the nerve endings. This was put to the test by assaying the acetylcholine output from a piece of intestine in the presence of eserine. Results obtained on the output of acetylcholine are shown in Fig. 14 and Table II. Fig. 14 gives part of an assay showing the reduction of the acetylcholine output by the presence of morphine in a concentration of 4×10^{-7}. Table II summarizes the effects of morphine on the output of acetylcholine at various frequencies, in three other experiments. In each of these, the output due to stimulation has been obtained by subtracting the resting output,

TABLE II

OUTPUT OF ACETYLCHOLINE FROM STIMULATED GUINEA-PIG ILEUM AT VARIOUS RATES OF STIMULATION, AND THE EFFECT OF MORPHINE

(ng./shock, corrected for resting output)

	(*a*)	(*b*)			(*c*)			
Rate of stimulation	50/min.	1/sec.	6·6/sec.	27/sec.	3½/min.	20/min.	1/sec.	5/sec.
Control	0·25	0·11	0·049	0·044	0·40	0·16	0·11	0·075
*Morphine treated	0·034 (10^{-6})	— (2×10^{-6})	0·050 (2×10^{-6})	0·042 (2×10^{-6})	— (4×10^{-6})	0·062 (4×10^{-6})	0·095 (4×10^{-6})	0·067 (4×10^{-6})

* Morphine concentration is given by the numerals in brackets.

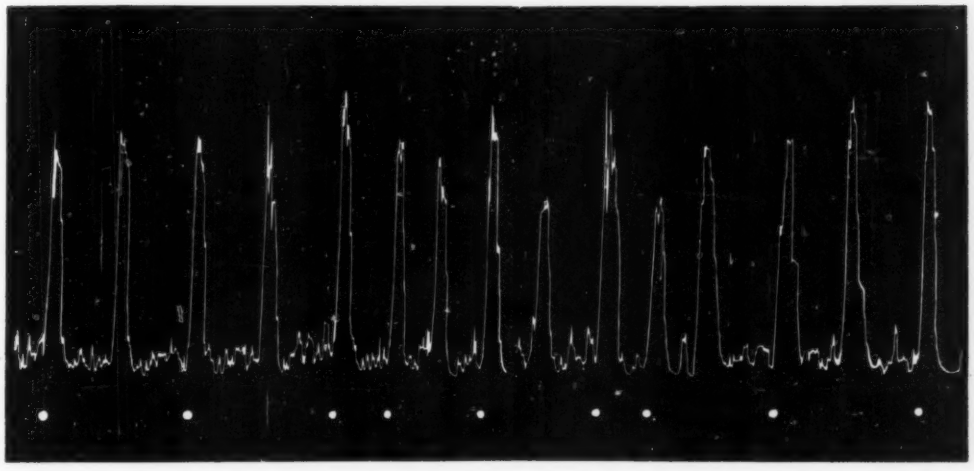

FIG. 14.—Reduction of acetylcholine output from stimulated eserinized guinea-pig ileum by morphine; part of the assay on another strip of ileum. Standard acetylcholine injections marked by white dots. Samples of fluid from donor bath (0.3 ml. = 1/25 total donor bath volume) during continuous stimulation at 5/sec., marked by black dots; successive samples taken every 3 min., the donor bath being then immediately and rapidly washed out. When morphine 0.4 μg./ml. was added to donor bath, the acetylcholine equivalent of sample fell from 3 ng. to 2 ng. After removing morphine, the acetylcholine equivalent returned to control level.

separately determined, from the total output observed. It will be seen here, as will be discussed more fully in another paper, that the normal output of acetylcholine decreases as the repetition frequency of the stimulus increases. But the effect of morphine is such as to reduce the acetylcholine output to an approximately constant low amount. Thus the proportionate reduction of acetylcholine output is greater on the slow rates of stimulation than on the rapid. It appears as though there were a morphine-susceptible quantum of acetylcholine output, together with an irreducible quantum which morphine cannot touch.

Morphine reduces the resting output by 20 to 40%; thus in three experiments the output fell from 2.7 to 1.5, 1.7 to 1.4, and 1.2 to 0.8 ng./min. respectively, in the presence of morphine in concentrations from 10^{-6} to 4×10^{-6}. It was also found, however, that sometimes the resting output increased above its initial level after the morphine was washed out; in the most striking case, the resting output, after stimulation for 20 min. at 5/sec. in the presence of morphine 2×10^{-6}, rose to 3.2 ng./min., from a control value of 1.1 ng./min.

It may be questioned whether the reduction of output of acetylcholine achieved by morphine is sufficient to produce failure of neuromuscular transmission in the intestine. It has already been stated that the output of acetylcholine/shock can be reduced in another way, by stimulating the intestine more rapidly. If this is done in the uneserinized preparation the twitch height begins to fall below its normal level at rates of about 20/min., and falls further as faster rates are applied. Combining this result with those of Table II, it may be estimated that failure of transmission will begin when the output falls below about a value of the order of 0.2 ng./shock. It is clear from the same table that morphine can reduce the output far below these levels.

It appears, therefore, that the action of morphine can be satisfactorily explained by a depression of acetylcholine output by it. But if acetylcholine output is depressed in this way one might expect that a similar depression of output could be obtained at other cholinergic synapses, such as the junction of motor nerves with striated muscle, or in autonomic ganglia. Accordingly the effect of morphine on acetylcholine output from the perfused ganglion was examined. It was found, however, as Hebb and Konzett (1949) reported, that morphine does not depress ganglionic transmission except in very large doses; indeed its first action is to potentiate the effects of preganglionic stimulation. In addition, a dose of 1 mg. injected into the arterial perfusion cannula had no effect on acetylcholine output. Similarly a test of morphine on the neuromuscular junction showed that it has singularly little neuromuscular effect; it required 5 mg. morphine given by close-arterial injection into the tibialis muscle of the cat to produce a very transient reduction of the twitch to sciatic nerve stimulation. It is clear, therefore, that the action of morphine is not exerted at all cholinergic synapses; the experiment on the ganglion serves to show, however, that when acetylcholine output is *not* depressed, transmission is not impaired.

Discussion

These experiments show that morphine, in low concentrations, has a depressant action on the postganglionic cholinergic nerve fibres of the guinea-pig intestine, reducing both the resting output of acetylcholine and the output from the nerve-endings when they are stimulated. In these concentrations, morphine did not stimulate the gut, nor interfered with its contractile power, nor lessened its sensitivity to acetylcholine. On this preparation, therefore, the action of morphine was exerted only on the nervous tissue and not on the smooth muscle. The work reported by Schaumann *et al.* (1952) and Schaumann (1955), using a different method, points to the same conclusion. Schaumann *et al.* showed that a number of strong analgesics could depress the peristaltic reflex of the guinea-pig ileum; and this was followed by the demonstration (Schaumann, 1955) that morphine could paralyse the preparatory phase of the peristaltic reflex; this phase of the reflex resists ganglion-blocking agents, but is sensitive to atropine, and so presumably involves postganglionic cholinergic nerve fibres. Kosterlitz and Robinson (1955) have confirmed these observations, and found in addition that morphine has an inhibiting effect on the action of 5-hydroxytryptamine, whose action on the ileum is now believed to involve a nervous pathway (Robertson, 1953; Rocha e Silva, Valle and Picarelli, 1953; Gaddum and Hameed, 1954).

No obvious reason can yet be advanced why this action of morphine is exerted on cholinergic nerve fibres in the intestine, but not on those of the autonomic ganglion or neuromuscular junction of striated muscle. It may be, indeed, that the action concerned is not one specifically on acetylcholine output, but that morphine has some other effect at the terminations of postganglionic nerves, which results in a failure of normal nerve terminal activity. Kosterlitz and Robinson (1955) suggest

that morphine may act on the interstitial cells of Cajal. This possibility cannot, at present, be tested by direct experiment. The failure of morphine to reduce the response of the gut to acetylcholine directly injected must exclude, on this theory, any rôle of the interstitial cells in the response of the gut to acetylcholine once it is free in the tissue spaces. If the action of morphine is to depress acetylcholine output, then the hypothesis would require that the interstitial cells were involved in the process of acetylcholine output. The postganglionic axones seem far the most likely source of acetylcholine, but it is not impossible that the interstitial cells serve as " moderators " of the nerve-terminal acetylcholine release.

The notion that morphine can act directly on the local nervous apparatus of the intestine, already proposed by Schaumann *et al.*, is relatively novel, and it probably cannot explain all the intestinal actions of morphine in the whole animal. It contributes only to a failure of propulsion by the intestine, together with any secondary results of such failure. But the spasmogenic action of morphine in the whole animal, distinct though possibly overestimated, must receive some other explanation, either by direct action on smooth muscle in some species, or by some central stimulant action with a visceral efferent component. Two other processes might contribute to an increased activity of the intestine after morphine, although they can hardly account for all of the results reported. In the first place, morphine is a histamine-liberator (Feldberg and Paton, 1951 ; Evans, Nasmyth and Stewart, 1952), and it is known that histamine-liberators can increase the motility of the intestine, although only with large doses can a frank contraction be produced (Feldberg and Smith, 1954). Secondly, in the experiments reported above, it was noticed that sometimes, following a sustained exposure to morphine, and after the morphine was washed out, the *resting* acetylcholine output increased ; whether this represented an increased leakiness of the cholinergic nerve endings, or a change in the output of acetylcholine from non-nervous sources, it may contribute to the enhanced though rather incoordinate motility of the gut produced sometimes by morphine administration.

All the analgesics tested had a morphine-like action. An important point was that those compounds more active than morphine as analgesics were also more active against the intestinal twitch ; and those less potent as analgesics were less active. This parallelism naturally suggests that the guinea-pig intestine provides a test-object for analgesics with closer analogies to the central nervous system than might be expected. This is borne out by a number of other observations. First, the concentrations of morphine required for activity compare well with those used therapeutically ; the threshold concentration for a distinct effect on the ileum was about 10 ng./ml. ; a dose, clinically, of ¼ grain to a subject weighing 75 kg. would, if dissolved in the whole body, give a concentration of 200 ng./ml., but lower concentrations probably exist in practice. Secondly, tolerance of the intestine to morphine could be readily produced, such that very large increments of morphine dosage produced dwindling actions. It was even possible to produce a state which could be interpreted as " morphine-dependence," whereby withdrawal of morphine from a tolerant strip of intestine would reduce its contraction in response to nerve-stimulation, and replacement of morphine restored it. Third, it is worth noting that the proportionate effect of morphine is less as the rate of excitation of the nerves is increased. This may well correspond to the clinical impression that severe pain can break through morphine analgesia.

It is striking how far the nervous system of the gut, the most nearly " autonomic " of all the peripheral nervous tissue, corresponds in its reactions to those of the central nervous system ; and it suggests that the enteric autonomic network furnishes a valuable simple paradigm of the far more complicated networks of the brain itself.

References

Evans, A. O. J., Nasmyth, P. A., and Stewart, H. C. (1952). *Brit. J. Pharmacol.*, **7**, 542.

Feldberg, W., and Paton, W. D. M. (1951). *J. Physiol.*, **114**, 490.

—— and Smith, A. N. (1954). Ibid., **124**, 219.

Gaddum, J. H., and Hameed, K. A. (1954). *Brit. J. Pharmacol.*, **9**, 240.

Hebb, C. O., and Konzett, H. (1949). *Nature* (*Lond.*), **163**, 720.

Kosterlitz, H., and Robinson, J. A. (1955). *J. Physiol.*, **129**, 18*P*.

Paton, W. D. M. (1955). Ibid., **127**, 40*P*.

——(1956). *Abstr. 20th int. physiol. Congr., Bruxelles*, 708.

Robertson, P. A. (1953). *J. Physiol.*, **121**, 54*P*.

Rocha e Silva, M., Valle, J. R., and Picarelli, Z. P. (1953). *Brit. J. Pharmacol.*, **8**, 378.

Schaumann, O., Giovannini, M., and Jochum, K. (1952). *Arch. exp. Path. Pharmak.*, **215**, 460.

Schaumann, W. (1955). *Brit. J. Pharmacol.*, **10**, 456.

Vaughan Williams, E. M., and Streeten, D. H. P. (1950). Ibid., **5**, 584.

—— —— (1951). Ibid., **6**, 263.

Brit. J. Pharmacol. (1957), **12**, 128.

SOME PHARMACOLOGICAL ACTIONS OF CHOLINE 2:6-XYLYL ETHER BROMIDE

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The following pharmacological properties of choline 2:6-xylyl ether bromide are described: (1) nicotine-like stimulant action on the arterial blood pressure, on the nictitating membrane, and on the soleus and gastrocnemius muscles of cats; (2) muscarine-like action on the arterial blood pressure of cats, and on the isolated guinea-pig ileum; (3) antihistaminic and antimuscarinic actions on the isolated guinea-pig ileum; (4) antisympathomimetic action on the arterial blood pressure of cats; and (5) neuromuscular blocking action on skeletal muscle of cats.

Hey and Willey (1953, 1954) described the long-lasting local anaesthetic action of choline 2:6-xylyl ether bromide (TM 10). They also described its nicotine-like stimulant action on the arterial blood pressure, and on the nictitating membrane, when given intravenously to atropine-treated cats (an action not obtained with subsequent doses); its transient blocking action of the pressor effect of adrenaline; and its action in abolishing the responses of the nictitating membrane to stimulation of its postganglionic nerve trunk. Brown and Hey (1956) have shown that this and related compounds inhibit the enzymic destruction of adrenaline by guinea-pig liver.

In view of the renewed interest in choline 2:6-xylyl ether (Exley, 1956; Bain and Fielden, 1956; Edge, Mason, and Wyllie, personal communication), it seemed worth while to put on record some observations that were not mentioned in the previous papers. These relate to the nicotine-like stimulant, neuromuscular blocking, muscarine-like, antisympathomimetic, antihistaminic, and antimuscarinic actions of the compound.

METHODS

Nicotine-like stimulant activity was tested by standard methods on the blood pressure of spinal cats and on the nictitating membrane and skeletal muscle of cats anaesthetized with chloralose (100 mg./kg., i.v.). Contractions of the soleus and gastrocnemius muscles were recorded with a Brown–Schuster mammalian-muscle myograph. Drugs were injected into the cannulated stump of the cut anterior tibial artery during occlusion of the popliteal artery.

Muscarine-like action was tested on the blood pressure of atropine-free cats anaesthetized with chloralose, and on the isolated guinea-pig ileum suspended in a 10 ml. bath of Tyrode solution at 35° C.

Antisympathomimetic activity was estimated on the blood pressure of spinal cats. The responses to doses of adrenaline and noradrenaline were determined before and after intravenous doses of TM 10.

Antihistaminic and antimuscarinic actions were studied on the isolated guinea-pig ileum.

Neuromuscular blocking activity was studied on the gastrocnemius muscle of anaesthetized cats. The sciatic nerve was stimulated by periodic condenser discharges (4/min.). Drugs were injected into the femoral vein.

RESULTS

Nicotine-like Stimulant Activity

Blood Pressure. — Intravenous injection of TM 10 to atropine-treated cats produces a brief rise in the arterial blood pressure, a characteristic effect of these nuclear-substituted choline phenyl ethers. But this effect of TM 10 is seen only on the first injection of the compound to an animal (Hey and Willey, 1954). Consequently, in assaying the activity of TM 10 against choline phenyl ether, only one estimation can be made on any one animal.

TM 10 is a very weak nicotine-like stimulant compound. It has, on a molar basis, approximately one-hundredth the activity of choline phenyl ether. This corresponds to one-half the activity of acetylcholine.

Despite the long-lasting abolition of the pressor responses to TM 10 that follows the first injection of this compound, the pressor responses to choline

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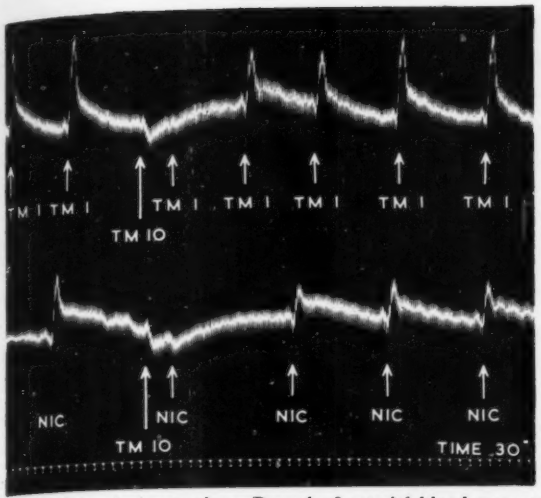

FIG. 1.—Cat, spinal, atropine. Record of arterial blood pressure showing transient blocking effect of TM 10 (10 mg., i.v.) on the pressor responses to i.v. injection of 50 μg. choline phenyl ether bromide (TM 1) and 0.5 mg. nicotine acid tartrate (Nic). Note the absence of presser response to TM 10 itself, owing to the previous injection (not shown on the record) of 10 mg. of the compound.

phenyl ether and to nicotine are only abolished when injected immediately after TM 10. This is illustrated in Fig. 1. The first injection of TM 10 (not shown in the record) had caused the usual marked pressor response. Subsequent doses of TM 10 no longer raise the blood pressure. The pressor responses to choline phenyl ether and to nicotine, injected immediately after the TM 10, are, however, completely abolished. Recovery then occurs fairly rapidly, the pressor response to choline phenyl ether returning to its original level, whilst a reduction in the response to nicotine is still apparent. (Since the first injection of TM 10 —the one that caused a pressor response—is not shown in Fig. 1, it must be pointed out that there was no lasting change in the responses to choline phenyl ether and to nicotine before and after that injection.) Thus, though TM 10 blocks its own pressor response for a long time, it causes only a transient block of the responses to choline phenyl ether and nicotine. This would suggest that TM 10 differs from the other two drugs in the way in which it produces its initial pressor effect. Nevertheless, it is known that the stimulant action of TM 10 is exercised, at least in part, at autonomic ganglia (see below).

Nictitating Membrane.—Hey and Willey (1954) showed that TM 10 causes a contraction of the nictitating membrane—an effect that is abolished by the removal of the superior cervical ganglion. The contraction evoked by TM 10, however, becomes progressively smaller with subsequent injections, until finally no contraction is produced.

The effect of TM 10 on the contractions of the nictitating membrane caused by electrical stimulation of the cervical sympathetic nerve, by nicotine, and by adrenaline, is shown in Fig. 2. Injection of 5 mg. TM 10 during electrical stimulation first reduces and finally abolishes the response. The

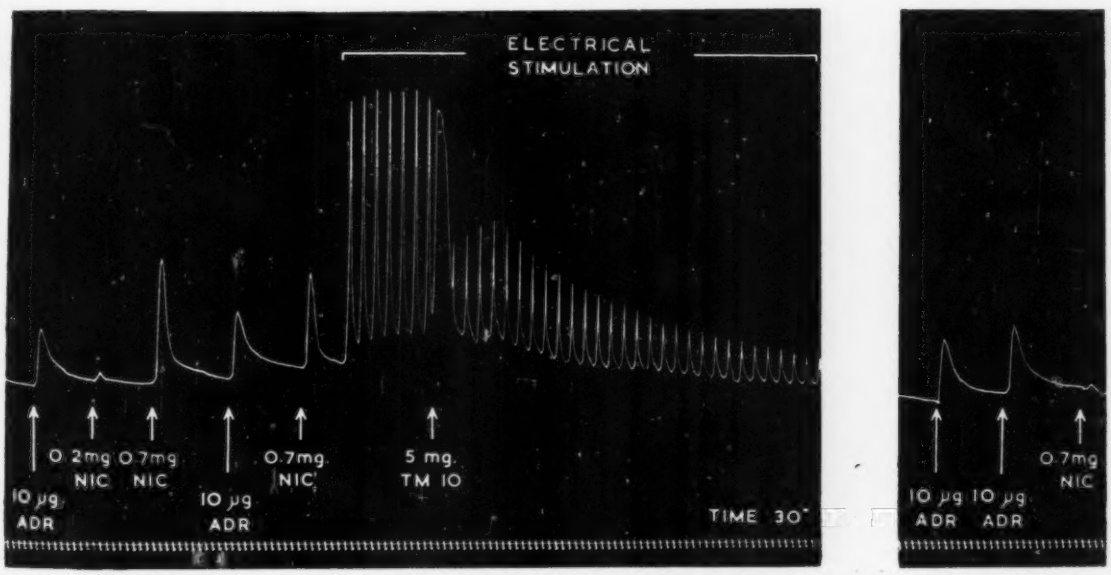

FIG. 2.—Cat, atropine, chloralose. Effect of TM 10 (5 mg., i.v.) on responses of the nictitating membrane to intravenous injections of 10 μg. adrenaline (Adr), and of 0.7 mg. nicotine acid tartrate (Nic); and to periodic electrical stimulation of preganglionic nerve trunk (70/sec. for 2 sec. in each min.). Time interval of about 30 min. between the two parts of the record.

stimulant effect of nicotine is also virtually annulled. The contraction produced by adrenaline is, however, unaffected.

In addition to the block of the response to preganglionic stimulation noted above, Hey and Willey (1954) showed that the response to postganglionic stimulation is also abolished by TM 10. It has already been mentioned that the contraction of the nictitating membrane evoked by TM 10 is abolished by previous administration of the drug; this is an effect analogous to that obtained on the arterial blood pressure. However, the effect of nicotine on the blood pressure is abolished for a much shorter time than is the corresponding effect on the nictitating membrane.

These curious differences between choline 2:6-xylyl ether on the one hand, and choline phenyl ether and nicotine on the other, are clearly worthy of systematic investigation.

Skeletal Muscle.—When injected into the cannulated stump of the cut anterior tibial artery

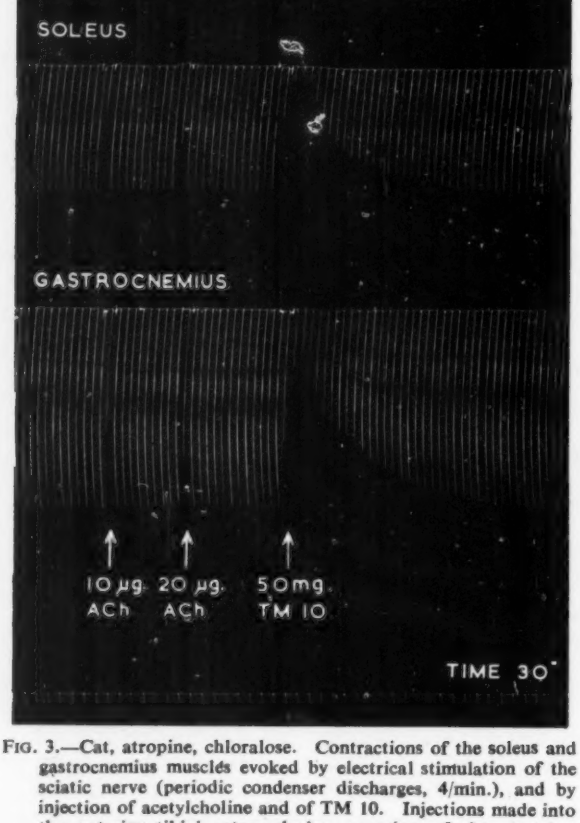

FIG. 3.—Cat, atropine, chloralose. Contractions of the soleus and gastrocnemius muscles evoked by electrical stimulation of the sciatic nerve (periodic condenser discharges, 4/min.), and by injection of acetylcholine and of TM 10. Injections made into the anterior tibial artery during cessation of the electrical stimulation. Note the transient neuromuscular blockade following the stimulant action of TM 10.

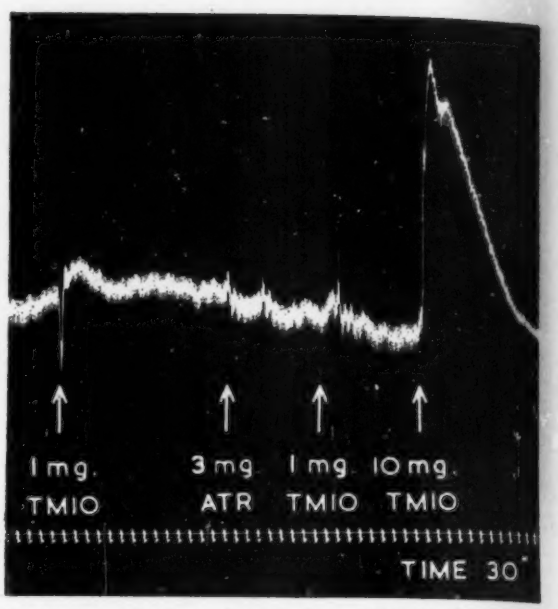

FIG. 4.—Cat, chloralose. Record of arterial blood pressure to show the abolition of the depressor response to TM 10 by atropine, and the pressor effect produced by a subsequent larger dose of TM 10.

TM 10 causes contractions of the soleus and gastrocnemius muscles. Fig. 3 shows the contraction produced by 50 mg. TM 10 compared with those produced by two different doses of acetylcholine. Applying this (2 + 1) assay technique, TM 10 was found to have approximately one five-thousandth the activity of acetylcholine as a stimulant of skeletal muscle. Fig. 3 also shows the transient neuromuscular blockade that follows the stimulant action of TM 10; this will be referred to again later.

Muscarine-like Activity

Intravenous injection of 1 mg. TM 10 causes a marked fall in the blood pressure of atropine-free cats under chloralose anaesthesia. This depressor response is abolished by the intravenous injection of 1 mg./kg. of atropine sulphate. This is illustrated in Fig. 4.

TM 10 also contracts the guinea-pig ileum in the absence of atropine. The contractions produced by TM 10 are not reduced by hexamethonium, in doses that abolish the contractions produced by nicotine (Fig. 5). Fig. 5 also shows that contractions produced by TM 10 and by acetylcholine are abolished by atropine sulphate in a concentration of 0.1 µg./ml. in the tissue bath. With concentrations of atropine that reduce, but do not abolish, the responses to acetylcholine and to TM 10 (20 ng./ml.), the reduction in the effect is the same for both drugs.

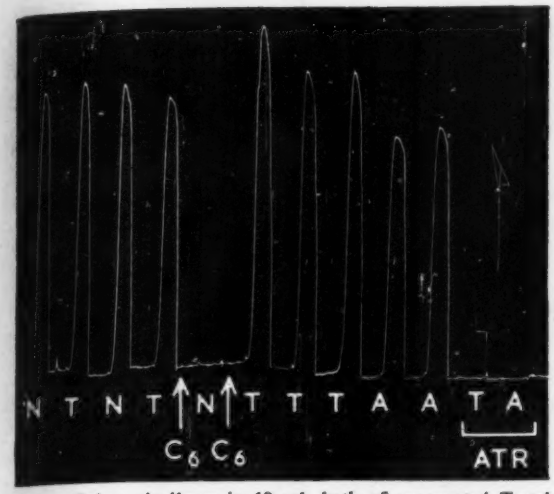

FIG. 5.—Guinea-pig ileum in 10 ml. bath of oxygenated Tyrode solution at 35° C. 4 min. cycle. N, 22 μg. nicotine; T, 40 μg. TM 10; A, 0.4 μg. acetylcholine chloride. In the presence of 4 mg. hexamethonium (C_6) the response to nicotine is abolished whereas the response to TM 10 is increased. Atropine sulphate, 0.1 μg./ml., during signal, abolishes the responses to acetylcholine and to TM 10.

The muscarine-like activity of TM 10 is approximately one-hundredth that of acetylcholine. But comparisons of TM 10 with acetylcholine are complicated. Thus the response to acetylcholine given immediately after TM 10 is frequently reduced—presumably because of an anti-acetylcholine action of TM 10 (Fig. 6). The effect of a subsequent dose of acetylcholine may, however, be increased; this may be accounted for by the weak anticholinesterase action of TM 10 (Willey, unpublished).

The doses of TM 10 generally used to produce this muscarine-like contraction in the isolated guinea-pig ileum ranged from 10 to 40 μg. in the bath. But in one experiment a large contraction was produced by 0.5 μg. TM 10. Repeated administration of the same dose led to progressively smaller responses. This phenomenon has been described by Ambache and Robertson (1953) for the 3-bromo- and 3:5-dibromo-phenyl ethers of choline, and was attributed by these authors to a nicotine-like paralysis.

Antihistaminic and Antimuscarinic Activities

Fig. 6 shows contractions of the isolated guinea-pig ileum to standard doses of histamine, acetylcholine, and barium chloride. Addition of 5 μg./ml. of TM 10 to the Tyrode solution caused a transitory contraction. In the presence of this concentration of TM 10, the response to the "musculotropic" spasmogen barium was unaffected, whereas the responses to acetylcholine and histamine were reduced, the latter to a more marked degree. Similar experiments, carried out with a higher concentration of TM 10 (20 μg./ml.), resulted in the complete abolition of the responses to histamine, whereas the responses to acetylcholine and barium were merely reduced.

It would appear, therefore, that TM 10, though possessing some general spasmolytic action, shows particular antimuscarinic and antihistaminic actions, more especially the latter. Estimations of the antihistaminic activity by Schild's method (Schild, 1947) gave mean pA_2 values of 5.03 (2 min. contact) and 4.98 (14 min. contact).

Antisympathomimetic Activity

In addition to the transient antagonistic action of TM 10 on the blood-pressure responses to adrenaline (Hey and Willey, 1954), the compound also blocks the pressor effect of noradrenaline. Fig. 7 shows the complete abolition of the blood-pressure responses to adrenaline and to noradrenaline after the intravenous injection of TM 10 to a spinal cat treated with atropine. Abolition of the responses, though complete for the doses used, is only transient; complete recovery occurs in about 30 min. (Fig. 7 also shows a potentiation of the responses to adrenaline and noradrenaline after the injection of TM 10—an

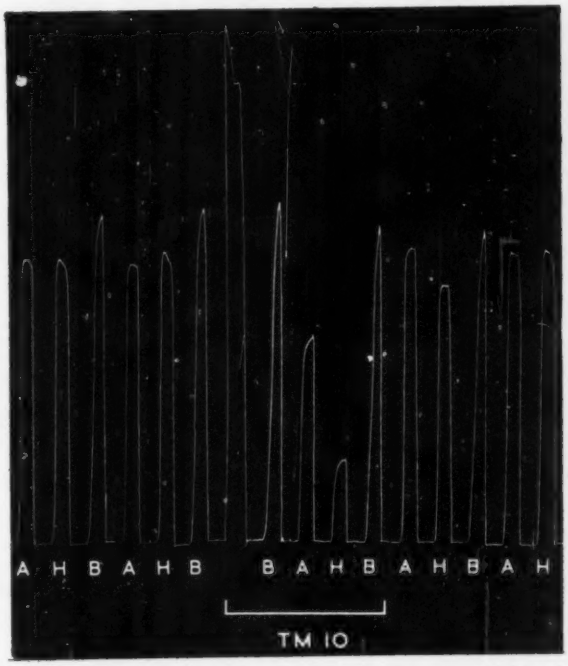

FIG. 6.—Guinea-pig ileum in 10 ml. bath of oxygenated Tyrode solution at 35° C. 4 min. cycle. A, 0.03 μg. acetylcholine chloride; H, 0.06 μg. histamine; B, 0.5 mg. barium chloride. TM 10, 5 μg./ml., during signal, reduces the responses to acetylcholine chloride and to histamine, but does not affect the response to barium chloride.

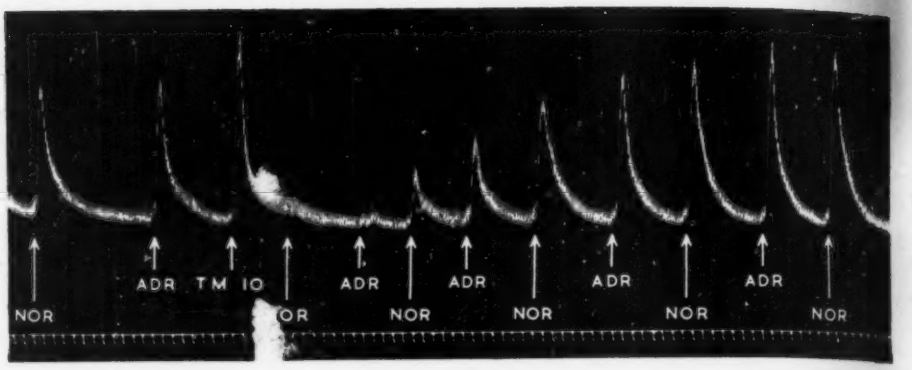

FIG. 7.—Cat, spinal, atropine. Record of arterial blood pressure to show the transient blocking effect of TM 10 (10 mg., i.v.) on the pressor responses to intravenous injections of 3 μg. adrenaline (Adr) and 1 μg. noradrenaline (Nor). Time signal, 30 sec.

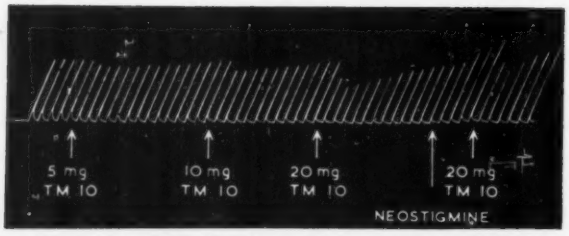

FIG. 8.—Cat, atropine, chloralose. Effect of intravenous TM 10 on isometric twitches of the gastrocnemius muscle evoked by electrical stimulation of the sciatic nerve (periodic condenser discharges, 4/min.). The block is unaffected by an injection of neostigmine methylsulphate (0.75 mg./kg.).

observation described by Brown and Hey (1956) for the related choline *p*-tolyl ether bromide.) The antisympathomimetic action of TM 10 has also been demonstrated on the nictitating membrane of the cat ; but, again, this action is of short duration, and adrenaline rapidly becomes effective again.

Neuromuscular Blocking Action

It has already been noted that TM 10 stimulates skeletal muscle, and then produces a transient neuromuscular blockade (Fig. 3). It has also been shown that the response to a close-arterial injection of acetylcholine is antagonized by TM 10.

In atropine-treated cats under chloralose anaesthesia, intravenous TM 10 causes a transient depression of the twitch tension of the gastrocnemius muscle stimulated through the sciatic nerve. This paralysis is unaffected by a preceding injection of neostigmine methylsulphate (Fig. 8). This last observation, taken together with the fact that TM 10 produces an initial stimulation of skeletal muscle (Fig. 3), suggests that the neuromuscular paralysis produced by TM 10 is of the decamethonium type.

I wish to thank Professor W. A. Bain for his advice and encouragement.

REFERENCES

Ambache, N., and Robertson, P. A. (1953). *Brit. J. Pharmacol.*, **8**, 147.
Bain, W. A., and Fielden, R. (1956). *J. Physiol.*, **133**, 70P.
Brown, B. G., and Hey, P. (1956). *Brit. J. Pharmacol.*, **11**, 58.
Exley, K. A. (1956). *J. Physiol.*, **133**, 70P.
Hey, P., and Willey, G. L. (1953). Ibid., **122**, 75P.
—— —— (1954). *Brit. J. Pharmacol.*, **9**, 471.
Schild, H. O. (1947). Ibid., **2**, 189.

ERRATUM

In the paper by P. J. Fraser in the March 1957 issue (Vol. 12, No. 1) an error appeared on page 48, col. 2, line 12. The total volume should read 3.0 ml. and not 3 ml.

Brit. J. Pharmacol. (1957), **12**, 133.

THE ACTIVITY OF DIFFERENT STEROIDS IN PRODUCING THYMIC INVOLUTION

BY

JENNIFER SHEWELL

From the National Institute for Medical Research, Mill Hill, London

(RECEIVED OCTOBER 17, 1956)

The assay method for corticotrophin, in which the involution of the thymus of the nestling rat is measured, can be applied successfully as a quick and simple screening test for biologically active corticosteroids. The sensitivity of the test is such that injection of a standard dose of cortisone acetate produces different results when made up in different media, or injected by different routes. A standard procedure for dispensing and injecting the steroids has therefore been adopted. Of the steroids investigated, it is noticeable that those possessing marked thymolytic activity are also steroids with therapeutic properties.

An attempt has been made to investigate the thymus-involuting activity of different steroids in relation to their molecular structure, as part of an investigation of the various biochemical properties of steroids, with particular reference to their effects on lymphoid tissue and circulating antibody.

It is generally accepted that the 11-oxysteroid hormones of the adrenal cortex will induce atrophy of lymphoid tissue in rats (White and Dougherty, 1946; Wells and Kendall, 1940; Ingle, 1950). It has been shown (Shewell, 1955; Shewell and Long, 1956) that animal species may be divided into two broad groups by their response to cortisone acetate. Species termed "cortisone-resistant" can withstand prolonged administration of cortisone acetate without losing body weight. Experimental animals, which, like man, are cortisone-resistant, are the guinea-pig and Rhesus monkey. Cortisone-sensitive animals, on the other hand, lose weight and undergo marked muscle wasting when cortisone acetate is administered: the rat, mouse, rabbit and ferret are cortisone-sensitive species. Although a certain degree of atrophy of lymphoid tissue is observed in cortisone-resistant species with cortisone administration, the effect in these species is not nearly as pronounced as that induced in cortisone-sensitive species. It was therefore decided to investigate the activity of steroids related to cortisone in producing involution of lymphoid tissue in the experimental animal found to be most sensitive to cortisone administration, namely, the rat. The thymus gland of the nestling rat was chosen as the most convenient organ to study, as representing mesenchymal tissue in general.

METHODS

Lymphoid tissue involution was measured by studying thymic atrophy. The thymus gland is readily removed by dissection, and consists almost entirely of lymphoid tissue: the sensitivity of its response to the administration of corticosteroids is much greater than that of the spleen (Fig. 1), a difference probably

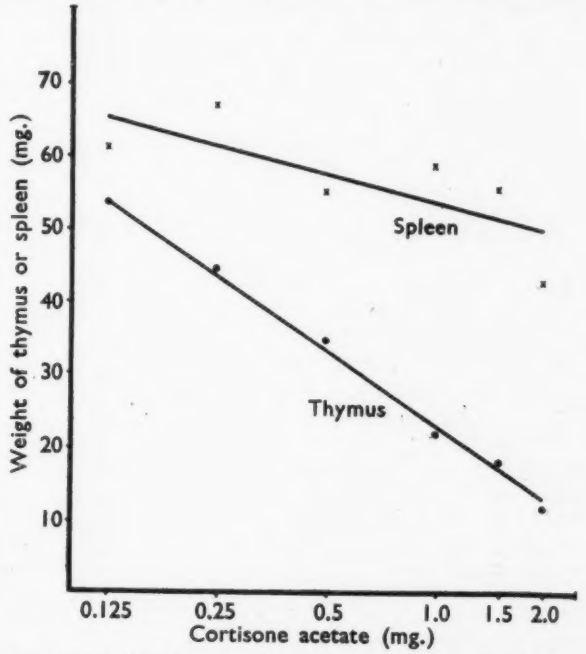

FIG. 1.—Log./dose response of spleen weight and thymus weight to cortisone acetate. Ordinates, weight of spleen and thymus in mg. Abscissae, dose of cortisone acetate on log. scale. Regressions fitted according to the method of least squares. The thymus provides a more accurate and sensitive index of the response of lymphoid tissue to the administration of cortisone acetate than does the spleen.

due to the fact that, among its other functions, the spleen is a blood-depôt. The method used was an adaptation of that developed by Bruce, Parkes, and Perry (1952) for the assay of corticotrophin in nestling rats. Animal variation was reduced by using litter-mate nestling rats of the hooded strain approximately 10 days old, weighing initially 12 to 15 g. Balanced groups of 8 rats were made up from 8 litters, so that one rat in each litter received one of the different treatments available, while at the same time the total weight of nestling rats in each treatment group was the same. The allocation of different treatments within the litter was made at random. One rat served as an untreated control, one as a saline-injected control, and six received doses of the unknown steroid and the laboratory standard steroid, cortisone acetate. The results were analysed as a (3+3) assay, plotting absolute thymus weight (mg.) against log. dose.

The nestling rats were marked with a colour for the litter, and a combination of site of colour and tail-mark for the individual members of the litter. For the next 3 days they were weighed and injected at the same time in each 24 hr. period, and on the fourth day they were weighed, and killed with chloroform. The thymus and spleen from each rat were dissected out and weighed fresh on a torsion balance. Since the administration of certain corticosteroids is known to produce increased glycogen deposition, with liver hypertrophy, the livers were also dissected out and weighed. This was done by clamping off the inferior vena cava with a Spencer-Wells forceps, and freeing the liver from its attachments without tearing the surface. The liver was gently blotted on gauze, and weighed fresh on a torsion balance. By standardizing the dissecting technique it was hoped to minimize errors in liver weight due to bleeding.

All steroid preparations were made up as saline suspensions, and 0.1 ml. of the different dilutions was injected under the loose skin in the rat's neck. A fine gauge (No. 18) needle was always used, to prevent leakage from the injection site. Cortisone acetate (Merck) was used as a laboratory standard. Preliminary studies of the effect of varying injection site and suspending vehicle had emphasized the necessity of comparing steroids under standard conditions. The estimations of comparative potencies in this paper refer to saline suspensions of the acetate injected subcutaneously as described above.

Results

When cortisone (Kendall's Compound E) and hydrocortisone (Compound F) were compared for their effects on thymus and liver weight, both as the alcohols and as acetates, it was found that neither of the alcohols was as effective in inducing either thymus atrophy or liver hypertrophy as was the acetate (Fig. 2). This was surprising, since clinical evidence (Boland, 1952) suggested that F-acetate was less effective in the treatment of rheumatoid arthritis than F-alcohol, and Porter

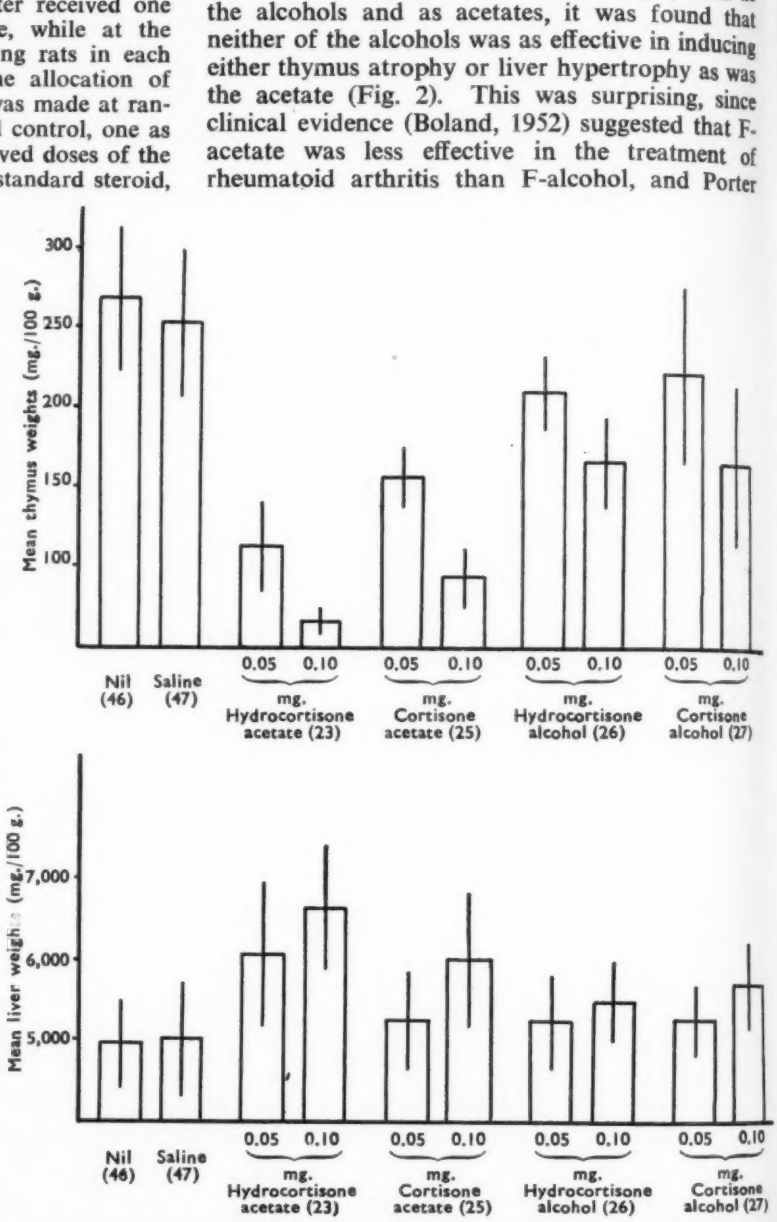

FIG. 2.—Thymic involution and liver hypertrophy produced by cortisone and hydrocortisone as the free alcohols and as acetates. The numerals in brackets give the number of rats in each group. The vertical bars represent the standard deviation from the mean. Both cortisone and hydrocortisone are more effective in inducing thymic atrophy and liver hypertrophy when given as acetates than when given as free alcohols.

and Silber (1953) found the alcohol to be more effective in promoting liver glycogen storage in the rat than hydrocortisone acetate.

It was thought that the relatively poor performance on the part of the alcohols might be due to differential absorption rates: if the alcohols were absorbed and eliminated very rapidly, the lasting concentration of steroids remaining in the blood would be too low to affect the thymus maximally. To delay absorption, therefore, cortisone alcohol and cortisone acetate were ground up in a beeswax-arachis oil mixture (the medium recommended by Bruce *et al.* (1952) to delay the absorption of corticotrophin) and injected subcutaneously into the neck as a 0.1% suspension. When the degrees of thymic involution and liver hypertrophy produced with this suspending agent are compared with those of the saline-suspended alcohol, a marked enhancement of activity occurred. Conversely, cortisone acetate which is absorbed at an optimal rate from a saline suspension is practically ineffective when made up in beeswax-arachis oil (Fig. 3).

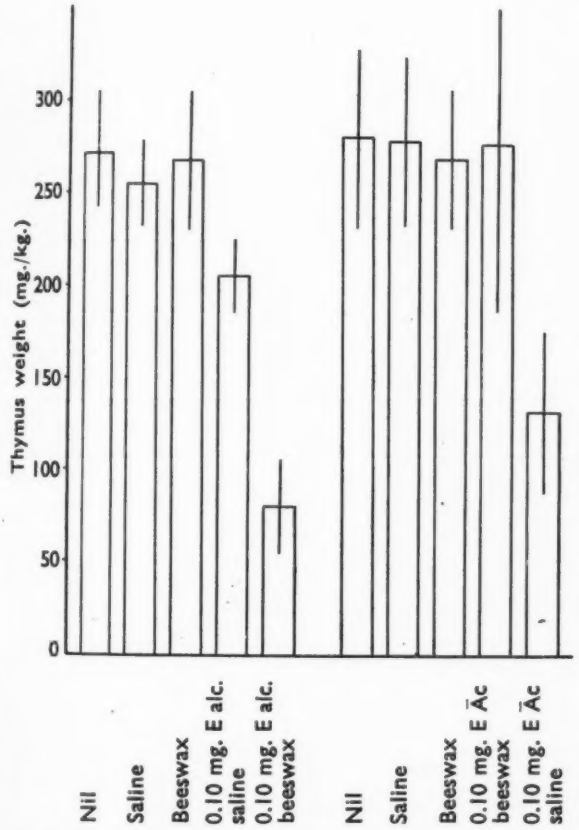

FIG. 3.—The effect of varying the suspending medium on thymic involution. The vertical bars represent the standard deviation from the mean. Cortisone (E) alcohol has only a small effect on thymus weight when made up in saline, while the same dose in beeswax-arachis oil produces much greater involution. On the other hand, cortisone acetate is effective in saline suspension, but has no effect on thymus weight when given in beeswax-arachis oil.

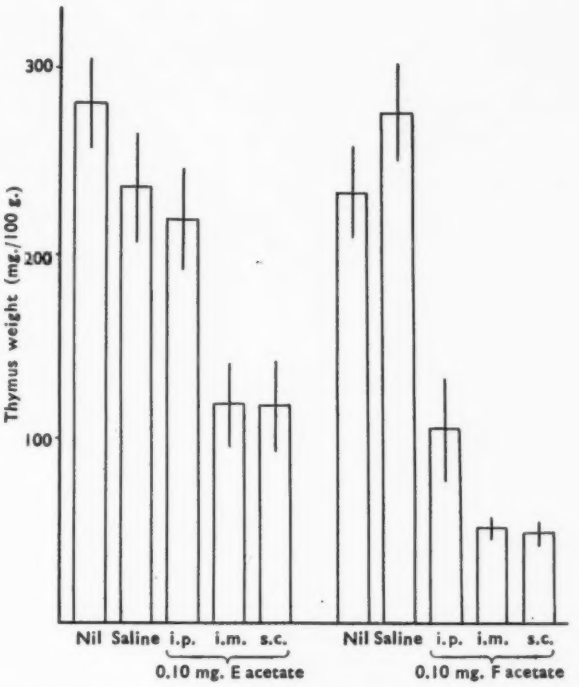

FIG. 4.—The effect of varying the injection route on thymic involution. The vertical bars represent the standard deviation from the mean. Cortisone (E) acetate and hydrocortisone (F) acetate are both less effective in producing thymic involution when injected intraperitoneally than when given either intramuscularly or subcutaneously. i.p., intraperitoneal; i.m., intramuscular; s.c., subcutaneous.

Differences in potency beween saline-suspended preparations when injected into different sites were also demonstrated. Cortisone acetate 0.1% is least effective in inducing thymic atrophy and liver hypertrophy when it is given intraperitoneally: slower absorption from a subcutaneous or intramuscular site results in greater effects on lymphatic tissue (Fig. 4).

These variations in effect produced by altering either the suspending vehicle or the injection site confirmed the importance of comparing all steroids to be tested under standard conditions. The relative potencies of the different steroids tested in producing thymic atrophy, compared with the standard preparation, cortisone acetate, are set out in Table I.

It will be seen that, as has previously been stated by Dougherty (1952), the criteria for thymolytic activity in the steroid molecule are the same as those for glucocorticoid activity, namely a double

TABLE I

THE RELATIVE POTENCIES OF DIFFERENT STEROIDS IN PRODUCING THYMIC INVOLUTION IN THE NESTLING RAT, AS COMPARED WITH CORTISONE ACETATE

All the steroids were tested as acetate preparations in saline suspension

Pregnane

Trivial Name	Systematic Name	Thymolytic Activity	Potency in Terms of Cortisone	5% Confidence Limits
Cortisone Kendall's Compound E	Pregn-4-ene-17α: 21-diol-3: 11: 20-trione	+	1	–
Hydrocortisone Kendall's Compound F	Pregn-4-11β: 17α: 21-triol-3: 20-dione	+	2·4	1·9– 3·0
9α-Chlorohydrocortisone ..	9α-Chloropregn-4-ene-11β: 17α: 21-triol-3: 20-dione	+	3·4	1·8– 6·3
9α-Fluorohydrocortisone ..	9α-Fluoropregn-4-ene-11β: 17α: 21-triol-3: 20-dione	+	5·3	4·2– 6·5
Prednisone	Pregna-1: 4-diene-17α: 21-diol-3: 11: 20-trione	+	9·9	6·6–14·6
Prednisolone	Pregna-1: 4-diene-11β: 17α: 21-triol-3: 20-dione	+	10·6	Calculated from assay against 9α-fluorohydrocortisone
9α-Fluoroprednisolone ..	9α-Fluoropregna-1: 4-diene-11β: 17α: 21-triol-3: 20-dione	+	13·2	9·8–17·0
*epi*Hydrocortisone	Pregn-4-ene-11α: 17α: 21-triol-3: 20-dione	–	–	–
Dihydro*allo*cortisone ..	*allo*Pregnane-17α: 21-diol-3: 11: 20-trione	–	–	–
Tetrahydrocortisone ..	Pregnane-3α:17α: 21-triol-11: 20-dione	–	–	–
Corticosterone Kendall's Compound B	Pregn-4-ene-11β: 21-diol-3: 20-dione	+	0·2	0·2–0·3
11-Dehydrocorticosterone Kendall's Compound A	Pregn-4-en-21-ol-3: 11: 20-trione	+	0·2	–
Reichstein's Substance S ..	Pregn-4-ene-17α: 21-diol-3: 20-dione	–	–	–
Pregnenetriolone	Pregn-4-ene-17α: 20β: 21-triol-3-one	–	–	–
Reichstein's Substance L ..	*allo*Pregnane-3β: 17α-diol-20-one	–	–	–
DOCA	Pregn-4-en-21-ol-3: 20-dione	–	–	–

bond at $C_{(4)}$, an oxo-group at $C_{(3)}$, and either an oxo-group or a β-hydroxyl group at $C_{(11)}$. Thus "*epi*-hydrocortisone," where the 11-hydroxyl group is in the α- and not in the β-position, has no effect on thymus weight; and both dihydro*allo*cortisone and tetrahydrocortisone, with no double bond at $C_{(4)}$, are inactive.

On the other hand, replacement of the hydrogen atom at $C_{(9)}$ by an α-halogen atom produced a molecule of greater thymolytic activity, together with increased ability to promote liver hypertrophy. 9α-Chlorohydrocortisone acetate is 3.4 times as active as cortisone acetate in causing thymus atrophy (Table I), and 4.8 times as active in promoting liver hypertrophy (Callow, Lloyd, and Long, 1954). An extra double bond in the A ring of the cortisone molecule, as in prednisone or prednisolone, increases thymolytic activity to 10 times that of the cortisone molecule. When the additional 1:2-double bond is combined with an α-fluorine atom at $C_{(9)}$, as in 9α-fluoroprednisolone, a molecule is produced with 13.2 times the thymolytic activity of cortisone acetate. This is not significantly greater, however, than the relative potencies of 10.6 and 9.9 shown by the unhalogenated prednisone and prednisolone molecules, so that there is no suggestion of any synergism between the thymolytic-activity-enhancing effects of the 1:2-double bond and the 9α-halogen atoms.

Some steroid compounds with one less oxygen atom in the molecule also exhibit thymolytic activity, although to a much lesser extent. Thus corticosterone, Kendall's Compound B, has approximately one quarter of the potency of cortisone acetate in producing thymus involution. Kendall's Compound A is indistinguishable from Compound B by this test.

The importance of the $C_{(11)}$ oxo- or β-hydroxyl-group is shown by the fact that neither Reich-

stein's Substances S or L, nor deoxycorticosterone acetate (DOCA), none of which has an oxygen atom at $C_{(11)}$, had any effect upon thymus weight.

Discussion

The thymus is a lymphoid tissue organ particularly susceptible to hormonal-induced atrophy (Weaver, 1955) and was therefore considered a suitably sensitive organ to investigate. A parallel investigation was carried out, through the courtesy of Professor G. R. Cameron, F.R.S., by Dr. V. Udall of the Department of Morbid Anatomy, University College Hospital Medical School, to ensure that it was the lymphoid tissue elements of the thymus that were affected by cortisone acetate, chosen as the standard laboratory steroid. Udall (1955) showed that thymus involution produced by cortisone acetate was apparently due to actual cell damage. Although he found no change in the number of cells in mitosis, the thymocyte count was significantly decreased, and the stem cell count significantly increased; he therefore suggested that, in addition to damaging the thymocytes, cortisone prevented the maturation of the stem cells.

In investigating experimentally-induced thymus atrophy, any possibility that experimental stress, resulting in an increased liberation of adrenocortical hormones, is producing thymic atrophy (Selye, 1950) must, of course, be ruled out. Dougherty and Santisteban overcame this difficulty (Santisteban, 1953; Santisteban and Dougherty, 1954) by studying "lympholytic" agents on the thymus of the adrenalectomized mouse. Their method involves surgery, which has two disadvantages: the number of animals in each experimental group has of necessity to be limited, and surgical procedures themselves stress the animals, so that thymus atrophy is measured after an earlier stress, unknown in extent, has been induced. It was found simpler to adapt the method of measuring thymus involution in the intact nestling rat developed by Bruce *et al.* (1952) for the assay of corticotrophin. This method utilizes Jailer's (1950) observations that the stress mechanism is not completely developed in the young rat, and Bruce *et al.* showed that any stress caused by handling and injecting young rats of up to 10 to 12 days old was insufficient to result in thymus atrophy. Thymic atrophy following injection of any steroid into the intact nestling rat may therefore be regarded as due to that steroid. Stephenson (1954, 1956) has used the thymus involution of 23-day-old weanling rats to compare the relative potencies of some adrenal steroids. While weanling rats have been used successfully in corticotrophin assays, it is felt that for comparing the action of different steroids the younger rat, with an incompletely developed stress mechanism, and with no question of sex difference affecting response, is to be preferred.

The vehicle in which the different steroid preparations are dispensed has been shown to affect the potency of the effect produced on the thymus. Stephenson (1954) considered that the rate of absorption of an 11-oxycorticosteroid from the injection site was inversely proportional to the degree of thymic involution produced, since hydrocortisone acetate given twice daily for three days to weanling rats was more effective in a sesame oil suspending medium than when made up in a 10% ethyl alcohol-saline mixture. This is, however, an over-simplification, since the absorption of cortisone acetate from a beeswax-arachis oil mixture is so slow that not maximal, but negligible, thymic involution takes place.

Similarly, alteration of the injection site, which also alters the rate of absorption of the steroid, is reflected in different potencies. The small degree of thymic atrophy produced by the intraperitoneal injection of cortisone acetate is in accordance with the results obtained by Greenspan, Gifford and Deming (1953), who were unable to influence thymus and adrenal weight in adult rats with intraperitoneal injections of cortisone acetate. They concluded that absorption and excretion had taken place too quickly to allow of any effect. An alternative explanation is that the steroids injected into the peritoneal cavity had been directly absorbed by the liver, and broken down there.

Among the steroids isolated from natural sources, the essential requirements for thymolytic activity are fulfilled only by cortisone, hydrocortisone, corticosterone and 11-dehydrocorticosterone (Kendall's Compounds E, F, B, and A). Of these, marked thymolytic activity is exhibited only by cortisone and hydrocortisone, which also are the only naturally occurring steroids to depress sensitivity to tuberculin in the guinea-pig (Long and Spensley, 1954) and to have any therapeutic effect in man in the treatment of rheumatoid arthritis. Any modification of the structure of the cortisone molecule, other than halogenation at $C_{(9)}$ or the introduction of a 1:2- double bond, results in a loss of thymolytic activity. Thus Kendall's Compounds B and A, with no hydroxyl group at $C_{(17)}$, but still with an oxo group at $C_{(11)}$, retain about one quarter of the thymolytic activity of cortisone. Flux (1954) has claimed thymolytic activity for Reichstein's Substance S. Studying

the effect of corticosteroids on the growth of mammary glands in mice, he weighed the thymuses from ovariectomized mice treated with different steroids and concluded that 2.0 mg. Substance S given daily to an 18 g. CHI mouse produced thymic atrophy. Thymic atrophy in an adult mouse with an intact pituitary-adrenal axis could quite possibly be an effect of stress due to injection technique. Since no involution was observed in the intact "unstressable" nestling rat, it seems most probable that Substance S has no thymolytic activity. No effect on thymus weight was observed with either Reichstein's Substance L or DOCA, although Santisteban and Dougherty (1954) state that deoxycorticosterone increased thymus size in the adrenalectomized mouse, when doses of 0.025 to 0.50 mg. were administered as the glucoside. Under the standardized conditions of the nestling rat experiments just described, doses of up to 0.20 mg./0.1 ml. of DOCA were without effect.

The synthesis of the halogenated cortisones and hydrocortisones by Fried and Sabo (1953) produced the first modification of the cortisone molecule with enhanced biological activity. Since varying only the suspending medium can alter effects in the rat thymus test, it is not surprising that other investigators, using widely differing animal tests, have obtained different relative potencies (Table II). The halogenated cortisone derivatives possess both mineralocorticoid and glucocorticoid activity, so that although clinically more potent than cortisone they are not suitable for use in treating rheumatoid arthritis, as they produce marked salt retention and oedema in man, but can be used to advantage in maintenance therapy after adrenalectomy (Hamwi and Goldberg, 1955).

The introduction of the 1:2- double bond in the A ring, producing prednisone, the unsaturated cortisone derivative, and prednisolone, the hydrocortisone derivative, was reported by Bunim, Pechet and Bollet in 1955. Again, different animal tests give differing values for relative potencies (Table II). The weight of clinical evidence shows that prednisone is approximately 3 to 4 times as effective as cortisone in the treatment of rheumatoid arthritis (Boland, 1956; Bollet, Black and Bunim, 1955; Dordick and Gluck, 1955; Gray and Merrick, 1955; Muller, 1955), about 5 times as effective in allergic disorders and asthma (Arbesman and Ehrenreich, 1955; Feinberg and Feinberg, 1956) and 4 times as effective in treating dermatoses (Robinson, 1955).

From the thymus involution test in the nestling rat, the degree of enhancement of biological activity produced either by halogenation or unsaturation of the cortisone molecule is greater than that borne out by clinical evidence. This is a reflexion of the sensitivity of the rat thymus test. It pro-

TABLE II

VALUES FOR RELATIVE POTENCIES OF THE HALOGENATED HYDROCORTISONES, PREDNISONE AND PREDNISOLONE, OBTAINED WITH DIFFERENT BIOLOGICAL TESTS

The standard steroid used is shown as E (cortisone alcohol), E $\bar{A}c$ (cortisone acetate), F (hydrocortisone alcohol) or F $\bar{A}c$ (hydrocortisone acetate)

Compound	Test	Potency	Author(s)
HALOGENATED HYDROCORTISONE DERIVATIVES			
9α-Chlorohydrocortisone acetate	Rat liver glycogen deposition	4 ×E $\bar{A}c$	Fried and Sabo (1953)
9α-Fluorohydrocortisone "	" " " "	10·7×E $\bar{A}c$	" " " (1954)
" "	" " " "	12·6×F $\bar{A}c$	Stafford, Barnes, Bowman, and Meinzinger (1955)
" "	Thymus involution weanling rat	8·8×F	Stephenson (1956)
" "	"Anti-inflammatory" effect round cotton pellet-rat	7 ×F	Dulin (1955)
" "	" " " "	13·2×F $\bar{A}c$	Singer and Borman (1956)
" "	Depression eosinophil count in dogs	20 ×F $\bar{A}c$	Liddle, Pechet, and Bartter (1954)
" "	Prevention Na^+ loss adrenalectomized dogs	4·7×DOCA	" " " " "
UNSATURATED CORTISONE AND HYDROCORTISONE DERIVATIVES			
Prednisone	"Anti-inflammatory" effect—rat hind paw	4·5×E $\bar{A}c$	Ducommun, Ducommun, and Baquiche (1955)
"	Rat liver glycogen deposition	3–4×E $\bar{A}c$	Bunim, Pechet, and Bollet (1955)
"	Granuloma pouch technique—rat	No different from E $\bar{A}c$	Demartini, Boots, Snyder, Sandson, and Ragan (1955)
"	Thymus involution weanling rat	2·96×F	Stephenson (1956)
Prednisolone	" " " "	4·28×F	" "
"	Rat liver glycogen deposition	2·9 ×F $\bar{A}c$	Stafford *et al.* (1955)
"	" " " "	10·1 ×E	Tolksdorf, Battin, Cassidy, MacLeod, Warren, and Perlman (1956)

vides a quick and easily carried out test for biological activity in different steroids; and while steroids with no clinical efficacy, such as corticosterone, produce slight thymic involution, the most effective involuting agents so far tested are those with therapeutic activity.

This work formed part of a thesis accepted by the University of London in part fulfilment of the requirements for the Degree of Doctor of Philosophy. It was carried out under the supervision of Dr. C. H. Andrewes, F.R.S., to whom I am most grateful. The work was done in Dr. D. A. Long's laboratory, and I am greatly indebted to both Dr. Long and to Dr. R. K. Callow for helpful advice. I am grateful to Miss M. V. Mussett, B.Sc., for carrying out the statistical analyses; and would like to thank Mrs. S. Brownstone and Mrs. W. Driver for skilled technical assistance.

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References

Arbesman, C. E., and Ehrenreich, R. J. (1955). *J. Allergy*, **26**, 189.
Boland, E. W. (1952). *Calif. Med.*, **77**, 1.
—— (1956). *J. Amer. med. Ass.*, **160**, 613.
Bollet, A. J., Black, R., and Bunim, J. J. (1955). Ibid., **158**, 459.
Bruce, H. M., Parkes, A. S., and Perry, W. L. M. (1952). *Lancet*, **1**, 790.
Bunim, J. J., Pechet, M. M., and Bollet, A. J. (1955). *J. Amer. med. Ass.*, **157**, 311.
Callow, R. K., Lloyd, Jennifer, and Long, D. A. (1954). *Lancet*, **1**, 20.
Demartini, F., Boots, R. H., Snyder, A. I., Sandson, J., and Ragan, C. (1955). *J. Amer. med. Ass.*, **158**, 1505.
Dordick, J. R., and Gluck, E. J. (1955). Ibid., **158**, 166.
Dougherty, T. F. (1952). *Physiol. Rev.*, **32**, 379.
Ducommun, P., Ducommun, S., and Baquiche, M. (1955). *Schweiz. med. Wschr.*, **85**, 1000.
Dulin, W. E. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 115.
Feinberg, A. R., and Feinberg, S. M. (1956). *J. Amer. med. Ass.*, **160**, 264.
Flux, D. S. (1954). *J. Endocrin.*, **11**, 238.
Fried, J., and Sabo, E. F. (1953). *J. Amer. chem. Soc.*, **75**, 2273.
—— —— (1954). Ibid., **76**, 1455.
Gray, J. W., and Merrick, E. Z. (1955). *J. Amer. geriatr. Soc.*, **3**, 337.
Greenspan, F. S., Gifford, H., and Deming, Q. B. (1953). *Endocrinology*, **52**, 638.
Hamwi, G. J., and Goldberg, R. F. (1955). *J. Amer. med. Ass.*, **159**, 1598.
Ingle, D. J. (1950). *J. clin. Endocrin.*, **10**, 1312.
Jailer, J. W. (1950). *Endocrinology*, **46**, 420.
Liddle, G. W., Pechet, M. M., and Bartter, F. C. (1954). *Science*, **120**, 496.
Long, D. A., and Spensley, P. C. (1954). *Lancet*, **1**, 645.
Muller, A. F. (1955). *Schweiz. med. Wschr.*, **85**, 1001.
Porter, C. C., and Silber, R. H. (1953). *Endocrinology*, **53**, 73.
Robinson, H. M. (1955). *J. Amer. med. Ass.*, **158**, 473.
Santisteban, G. A. (1953). *Anat. Rec.*, **115**, 366.
—— and Dougherty, T. F. (1954). *Endocrinology*, **54**, 130.
Selye, H. (1950). *Stress*, p. 452. Montreal, Canada: Acta Inc.
Shewell, J. (1955). "The influence of hormones on immunity." University of London: Ph.D. thesis.
—— and Long, D. A. (1956). *J. Hyg., Camb.*, **54**, 452.
Singer, F. M., and Borman, A. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 23.
Stafford, R. O., Barnes, L. E., Bowman, B. J., and Meinzinger, M. M. (1955). Ibid., **89**, 371.
Stephenson, N. R. (1954). *Canad. J. Biochem. Physiol.*, **32**, 689.
—— (1956). Ibid., **34**, 253.
Tolksdorf, S., Battin, M. L., Cassidy, J. W., MacLeod, R. M., Warren, F. H., and Perlman, P. L. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 207.
Udall, V. (1955). *J. Path. Bact.*, **69**, 11.
Weaver, J. A. (1955). Ibid., **69**, 133.
Wells, B. B., and Kendall, E. C. (1950). *Proc. Mayo Clin.*, **15**, pp. 133, 324.
White, A., and Dougherty, T. F. (1946). *Proc. nat. Acad. Sci., Wash.*, **46**, 859.

Brit. J. Pharmacol. (1957), **12**, 140.

EFFECT OF CHAIN LENGTH OF ALIPHATIC AMINES ON HISTAMINE POTENTIATION AND RELEASE

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Diamines in the series $NH_2.(CH_2)_n.NH_2$ specifically potentiate histamine contractions of the guinea-pig ileum and inhibit the enzymatic destruction of histamine. These activities are greatest with short-chain compounds ($n \simeq 5$). Diamines also release histamine from isolated tissues and depress the contractility of plain muscle and the motility of paramecia. These activities increase with chain-length and are probably limited only by solubility. The parallelism between histamine-releasing activity and toxicity also extends to the monoamines in the series $CH_3.(CH_2)_{n-1}.NH_2$. Histamine-releasing activity of both series increases with increase of *p*H and can mainly be attributed to the non-ionized base.

From the results on the effect of chain-length and ionization on activity, it is suggested that aliphatic amines release histamine by penetration of the cell membrane in the non-ionized form, followed by exchange in the ionic form with intracellular histamine.

Within the homologous series of monoamines $CH_3.(CH_2)_{n-1}.NH_2$ and diamines $NH_2.(CH_2)_n.NH_2$ are found compounds which are destroyed by diamine oxidase (Blaschko and Hawkins, 1950), inhibit the enzyme competitively (Zeller, 1942), potentiate the actions of histamine (Arunlakshana, Mongar, and Schild, 1954), and release histamine (MacIntosh and Paton, 1949).

This paper describes the influence of chain-length and *p*H on these properties. Part of the results have already been briefly described (Mongar and Schild, 1953a).

METHODS

Inhibition of histaminase was measured by incubating various concentrations of diamines with a crude histaminase preparation consisting of an acetone-dried powder of pig kidney cortex. Potentiation of histamine contractions of the guinea-pig ileum was estimated in terms of the equivalent histamine dose, taking the dose before adding the potentiator as unity. These methods have been described in detail (Arunlakshana *et al.*, 1954).

For the quantitative measurement of the depression of the motility of paramecia, the concentration of amine required to immobilize half the organisms in a given colony in 5 min. was determined. This was done by pipetting equal volumes of a suspension of *Paramecium aurelia* into six 2 ml. perspex cups fitted into the revolving stage of a low-powered microscope (Fig. 1). Each cup contained several hundred organisms. At zero time, a different concentration of amine was added to each cup and at intervals during the following 60 min. the colonies were repeatedly observed in turn by rotating the stage. Above a certain concentration all paramecia became immobilized within 1 min. Below 1/10 of this concentration the organisms were unaffected. With intermediate concentrations the fraction of paramecia still swimming about was estimated.

The concentrations of all amines when expressed on a w/v basis are given in terms of the chlorides.

RESULTS

Inhibition of Histaminase.—The activity of diamines of different chain lengths in inhibiting the destruction of histamine by histaminase is shown in Table I. Inhibition was measured by incubating histamine with histaminase in the presence of various concentrations of amines. The

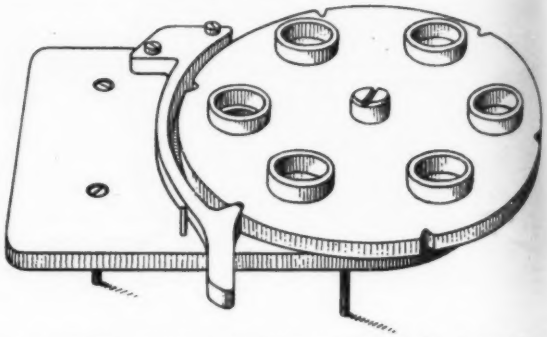


FIG. 1.—Rotating perspex microscope stage fitted with six 2 ml. cups and a locking device. The contents of each cup can be conveniently examined in quick succession.

TABLE I

INHIBITION OF HISTAMINASE BY STRAIGHT CHAIN ALIPHATIC DIAMINES

Histamine destruction by histaminase is inhibited by the presence of diamines. The table gives the reduction in the amount destroyed as % of that destroyed in controls containing no diamine. A different preparation of enzyme, in the form of acetone-dried pig kidney cortex, was used in each series of experiments. Histamine destruction in the controls was 60% in Series I and 80% in Series II.

	Chain Length	Concentration of Diamine				
		10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
Series I . .	C_5	Nil	11	4½	26½	49
	C_{10}	,,	Nil	6	18	10½
	C_{15}	4	,,	Nil	10	(insol.)
,, II	C_2	—	,,	4	7½	72
	C_4	—	,,	8	18½	86
	C_6	—	,,	2	—	40
	C_8	—	,,	1	10½	12

time of incubation was chosen to give 60 to 80% destruction of histamine in the absence of inhibitor. Table I gives % inhibition of destruction by various concentrations of diamines. As a group these compounds were weak inhibitors of histaminase, but their activity varied according to chain length. None produced an appreciable effect below a concentration of 1 mM. Concentrations producing 20% inhibition were determined, when necessary by extrapolation, from a plot of % inhibition against the logarithm of the concentration. Fig. 2 shows the pI_{20} values (Blaschko, Bülbring, and Chou, 1949). Inhibiting activity is maximal at about C_4 and falls off on either side. The C_{15} compound has only about 1/100th of the maximal activity.

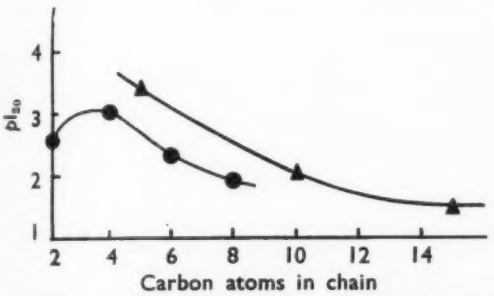


FIG. 2.—Activity of diamines as inhibitors of histaminase. The negative logarithm of the concentration required to produce 20% inhibition has been plotted against chain length for two series of experiments each with a different preparation of histaminase. Upper curve, series I diamines; lower curve, series II diamines (see Table I).

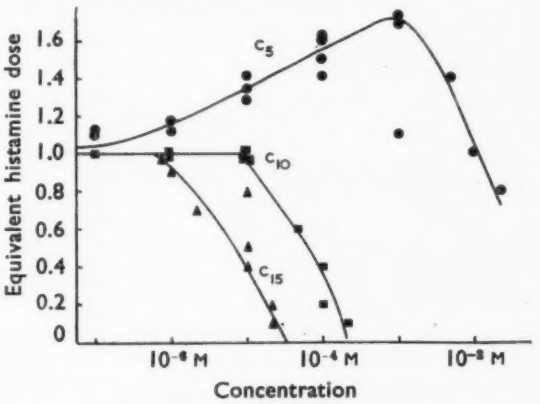


FIG. 4.—Potentiation and depression of histamine contractions of the guinea-pig ileum by various concentrations of diamines with chain lengths of 5, 10, and 15 carbon atoms.

Potentiation.—Fig. 3 shows the potentiation of the histamine contractions of guinea-pig ileum by cadaverine (pentamethylenediamine). When cadaverine was added to both washing and drug solutions it caused an immediate and persistent increase of the effect of histamine although it did not itself cause contraction of the ileum. Compounds of longer chain length, which produced only a weak inhibition of histaminase, did not potentiate but only antagonized histamine. Fig. 4

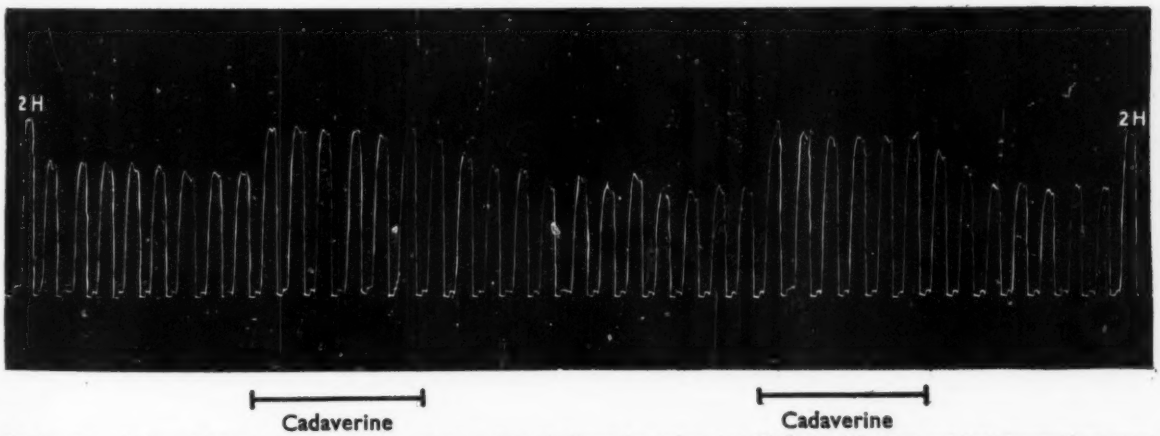


FIG. 3.—Potentiation of histamine contractions of the guinea-pig ileum by cadaverine (10^{-3} M). The first and last contractions were produced with a dose of 20 ng. histamine (2H) and the intermediate ones with 10 ng.

shows the effect of diamines with chain lengths of 5, 10, and 15 carbon atoms on histamine contractions of the guinea-pig ileum. The C_5 compound produced potentiation which increased with concentration up to 1 mM. and subsequently declined; high concentrations produce a depression. The C_{10} and C_{15} compounds produce only depression of histamine contractions. This depressant effect, which increased with chain length, is considered in more detail in a later section. The shapes of the curves in Fig. 4 can be explained in terms of the two opposing factors, potentiation and depression, both of which increase with concentration.

Histamine-releasing Activity.—The histamine-releasing activity of diamines has been demonstrated by MacIntosh and Paton (1949), by the test of the delayed fall in blood pressure in the cat anaesthetized with chloralose. The results reported have been obtained using minced guinea-pig lung (Mongar and Schild, 1953b), and isolated rat diaphragm (Rocha e Silva and Schild, 1949) to measure histamine-releasing activity of diamines of chain length C_5 to C_{15}. Fig. 5 shows the results

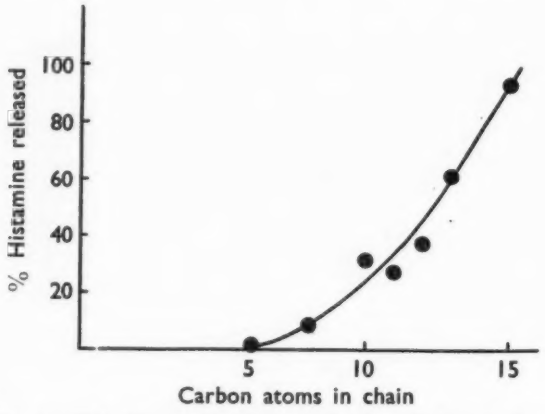


FIG. 5.—Histamine release from minced guinea-pig lung by 1 mM. solutions of straight-chain aliphatic diamines of various chain lengths. The amount released in 15 min. is expressed as % of the total tissue histamine.

obtained with equimolar solutions of diamines acting on minced guinea-pig lung. The activity increases steadily with chain length from less than 5% release with cadaverine to more than 90% release with pentadecamethylene-diamine during a test period of 15 min.

The effect of all diamines increased steeply with concentration. Fig. 6 shows that even cadaverine releases histamine in high concentrations. In a concentration of 1 mM. it is inactive both on minced guinea-pig lung and rat diaphragm, but at 100 mM. it releases about 80% of the histamine content of

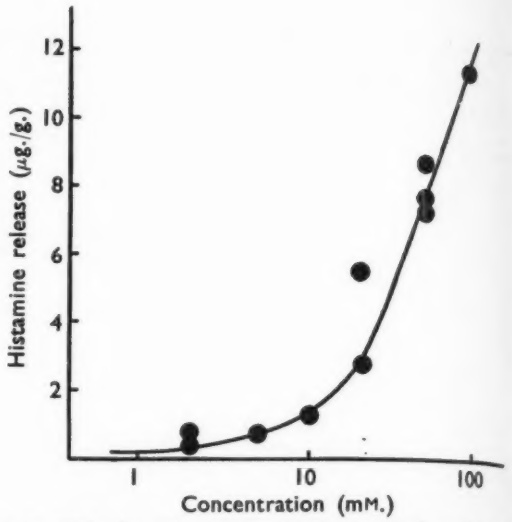


FIG. 6.—Histamine release in 10 min. from isolated rat diaphragm by various concentrations of cadaverine.

the tissue. The quantity of histamine released from minced guinea-pig lung in 60 min. by two concentrations of 5 different diamines is shown in Table II. From these results the concentrations of releaser giving 50% release in 60 min. were estimated, assuming a common regression coefficient. The values obtained are plotted against

TABLE II

PERCENTAGE OF HISTAMINE CONTENT OF GUINEA-PIG LUNG RELEASED IN 60 MIN. BY VARIOUS CONCENTRATIONS OF DIAMINES

Chain Length	Concentration of Diamine					
	$3{\cdot}2\times10^{-5}$	10^{-4}	$3{\cdot}2\times10^{-4}$	10^{-3}	$3{\cdot}2\times10^{-3}$	10^{-2}
C_5	—	—	—	—	45	75
C_8	—	—	45	64	—	—
C_{10}	—	—	53	102	—	—
C_{12}	—	56	68	—	—	—
C_{15}	31	64	—	—	—	—

chain length in Fig. 7*a*. In contrast to antihistaminase activity, histamine-releasing activity increases steadily with chain length.

Antihistamine Activity.—As shown in Fig. 4, high concentrations of diamines produce a depression of the histamine contractions of the guinea-pig ileum, and this depression increases with chain length. The antagonism for histamine has been determined quantitatively and the specificity of this antagonism has been investigated.

The antagonism measurements were done on the guinea-pig ileum; the antagonist was in contact for sufficient time to produce a steady depression. The concentrations of a number of diamines required to depress the effect of a double dose of histamine to that of a single dose were

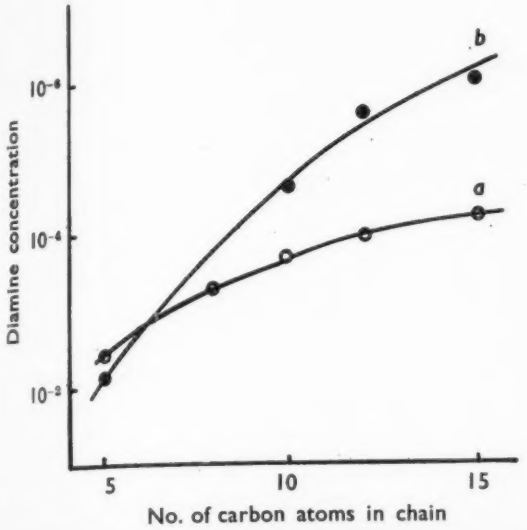
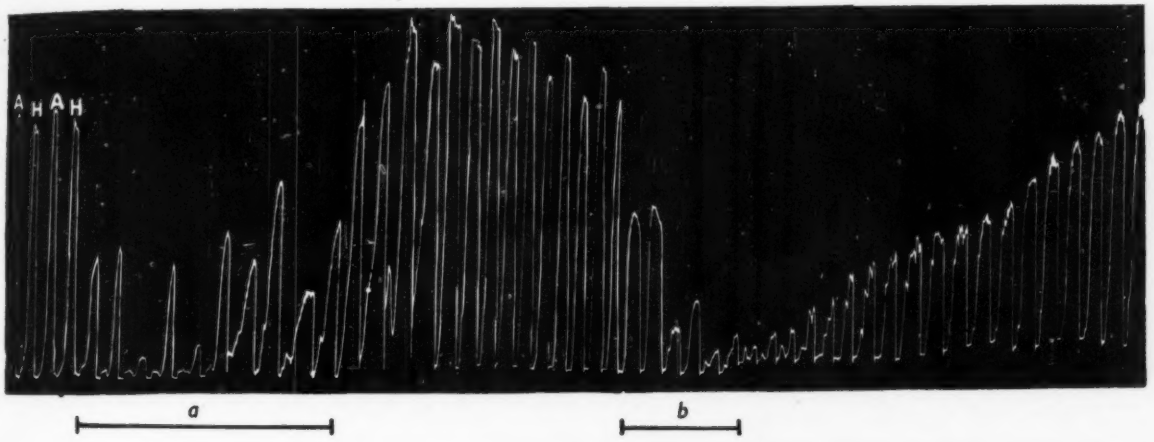


FIG. 7.—Effect of chain length on activity of diamines. *a*, Histamine release from guinea-pig lung: *b*, antagonism of histamine contractions of guinea-pig ileum.

determined (Fig. 7*b*). Antihistamine activity increases more steeply with chain length than the releasing activity; thus C_{15} is 7,000 times more active than C_5 as an antagonist but only 60 times more active as a releaser.

To determine whether this antagonism is specific, the antagonism towards acetylcholine was also measured. Experiments in which antagonism for histamine and acetylcholine was measured concurrently are illustrated in Fig. 8. Alternate equiactive doses of the two stimulant drugs were given. Following the addition of diamines C_{10} and C_{15} and the monoamine C_8 the effects of both stimulant drugs were depressed to roughly the same extent and after removal of the antagonist the contractions recovered at approximately the same rate. The responses to acetylcholine were slightly more reduced than those to histamine. These results suggest that the antagonistic effect of the amines is unspecific.

Toxicity to Paramecia.—Since the antagonistic effect of the long-chain amines was suggestive of



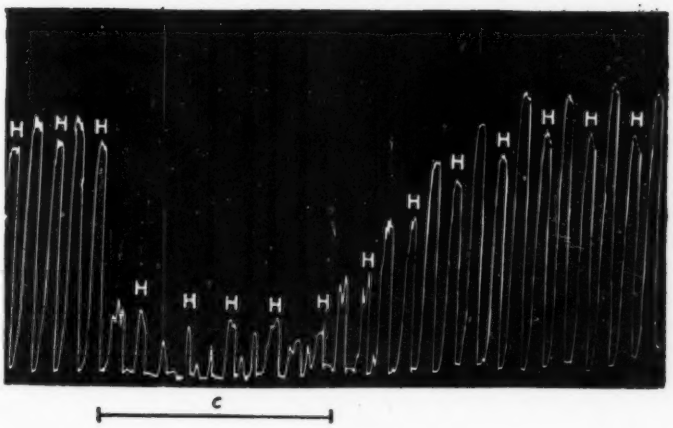


FIG. 8.—Depression by monoamines and diamines of contractions of guinea-pig ileum produced by alternative equiactive doses of histamine and acetylcholine. *a*, 2×10^{-5} $NH_2.(CH_2)_{10}.NH_2$; *b*, 10^{-6} $NH_2.(CH_2)_{15}.NH_2$, and *c*, 10^{-5} $CH_3.(CH_2)_7.NH_2$, were present in the bath fluid.

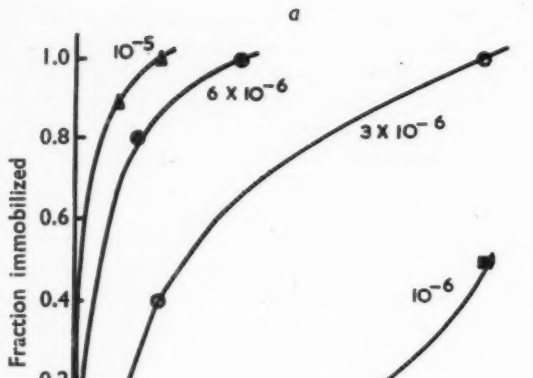


FIG. 9.—Characteristics of the paramecia toxicity test. *a*, Time action curves with various concentrations of decamethylene diamine. *b*, Concentration-action curve for a time of 5 min.

an unspecific depression of cell function rather than a specific antagonism, the toxicity of these compounds to unicellular organisms was also studied. A convenient measure was their activity in depressing the motility of paramecia. In order to obtain quantitative results the procedure illustrated in Fig. 9 was used. Fig. 9*a* shows the rate at which *P. aurelia* is immobilized by different concentrations of decamethylenediamine. By drawing a smooth curve through the points the fraction immobilized by a given concentration in a given time can be obtained. Fig. 9*b* shows the curve which results when this fraction is plotted against concentration of amine. From this curve the ED50 may be obtained by interpolation. The steep slope of the concentration-action curve (5% to 95% immobilization for a three-fold change of concentration) allows equiactive concentrations to be determined with a fair degree of accuracy.

Table III gives the concentration of various amines required to immobilize 50% of the organisms in 5 min. The activity of diamines increases steadily with chain length. The toxicity for paramecia of the monoamines also depends on chain length, but has a maximum at C_{12}.

TABLE III

TOXICITY OF AMINES TO PARAMECIA

The approximate concentrations required to immobilize 50% of the organisms in 5 min. are given. All concentrations are $\times 10^{-4}$

Monoamines		Diamines	
Chain Length	Conc.	Chain Length	Conc.
C_4	400	C_6	300
C_6	300	C_8	200
C_8	20	C_{10}	60
C_{10}	2	C_{12}	20
C_{12}	0·6	C_{13}	10
C_{16}	2·1	C_{16}	1

Comparison of Histamine-releasing Activity and Toxicity to Paramecia.—Fig. 10 shows the toxicity to paramecia of mono- and di-amines and also

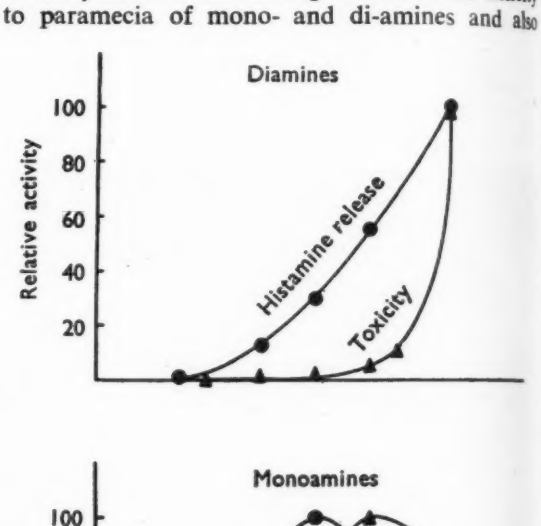


FIG. 10.—Effect of chain length on histamine-releasing activity and toxicity to paramecia for two series of aliphatic amines. The concentrations of the most active members of the series were: Monoamines: histamine release C_{10}, 7.2×10^{-5}; toxicity C_{12}, 0.6×10^{-5}. Diamines: histamine release 6.0×10^{-5}; toxicity 1.0×10^{-5}. The histamine-releasing activity is expressed in terms of the most active member of the series studied. The figures for histamine release by monoamines are from Mongar and Schild (1953b).

their histamine-releasing activity expressed in terms of the most active member of each series. The curves for histamine-release and toxicity are similar. Those for the diamines show a steady increase of activity with chain length with no suggestion of a maximum having been reached with the C_{15} compound. Those for the monoamines show a maximum by both tests which is probably due to micelle formation. Micelle formation does not occur with monoamines of 8 or less carbon atoms, but is known to occur with longer chains when a critical concentration is exceeded (Ralston and Hoerr, 1942). The critical concentration decreases with increase of chain length. The explanation of the maximum being due to micelle formation is borne out by the finding that the maximum of the curve for toxicity occurs at a greater chain length than the maximum for histamine release. Since lower concentrations are required to immobilize paramecia than to release histamine, the critical micelle concentration is reached earlier in the latter test.

Effect of p*H on Histamine-releasing Activity.*—Monoamines and diamines are both strong bases with pK_a of about 10.8 (Hoerr, McCorkle, and Ralston, 1943), and at *p*H 7 they are almost completely ionized. If the histamine-releasing activity of these solutions were due to the cation, change in *p*H would be expected to have little effect on activity. If on the other hand the histamine-releasing activity were due to the minute amount of non-ionized base present, an increase in *p*H would produce a marked increase in its concentration and hence would be expected to increase histamine release.

Fig. 11 shows the effect of *p*H on the histamine-releasing activity of octylamine and decamethylenediamine. The compounds are inactive at *p*H 7, but their activity rises sharply with *p*H to reach a very high value at *p*H 8.5. The question arises whether the increased histamine-release in alkaline solution is due to a change in ionization of the histamine-releaser or to an action on the tissue itself. The following findings suggest that the tissue itself is not involved: (i) There is no appreciable histamine release in Tyrode solution between *p*H 6.5 and 8.5. (ii) When a histamine-releaser is used whose ionization is not affected by *p*H, such as the quaternary compound dodecyltrimethylammonium chloride, histamine-releasing activity does not increase appreciably with *p*H. These results suggest that the striking effect of *p*H on the activity of the primary amines is due to a change in ionization of the releaser.

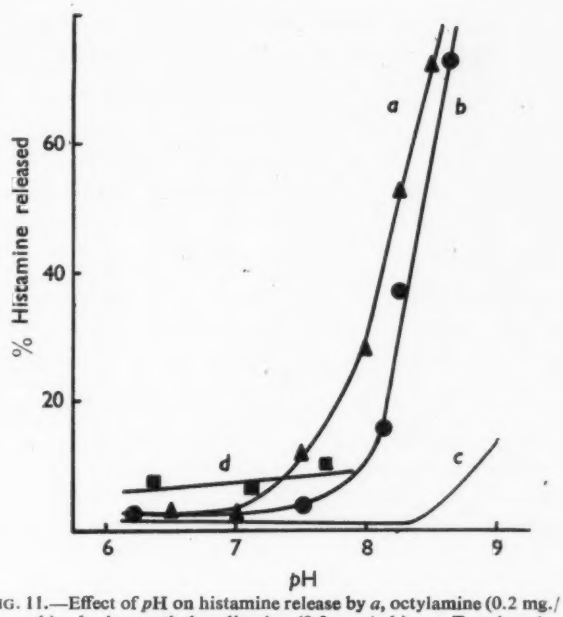


FIG. 11.—Effect of *p*H on histamine release by *a*, octylamine (0.2 mg./ml.); *b*, decamethylenediamine (0.2 mg./ml.); *c*, Tyrode solution; *d*, dodecyltrimethylammonium chloride (0.2 mg./ml.).

In a concentration of 0.2 mg./ml., the activity of octylamine and decamethylenediamine resides completely in the non-ionized form, since the histamine-releasing activity disappeared completely when the *p*H was reduced. If histamine-releasing activity were due solely to undissociated base, the *p*H-release curves obtained with different concen-

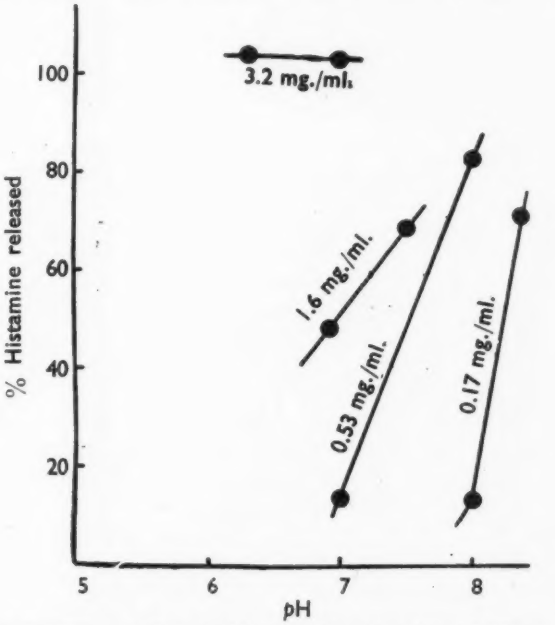


FIG. 12.—Effect of *p*H on the histamine-releasing activity of different concentrations of octylamine.

trations of the same releaser would be parallel. However, as shown in Fig. 12, this is not so. The curves obtained with different concentrations of octylamine are not parallel but become progressively less steep with increasing concentration of releaser. This means that as the concentration is increased the ionized molecules play an increasing part in histamine release and the contribution of the non-ionized molecules decreases. Fig. 13*a* shows the concentration of octylamine required to produce the same effect (50% histamine release) at different *p*H. From these results, and the pK_a of octylamine, the concentration of free base present at various *p*H's was calculated and is shown in Fig 13*b*. If the histamine-releasing activity resided entirely in the free base this curve would be horizontal. When the *p*H is high and the concentration of releaser is low the curve approaches the horizontal at a concentration of about 1 μg./ml. The free base thus appears to be highly active, producing a 50% histamine release at a concentration of 10^{-6}.

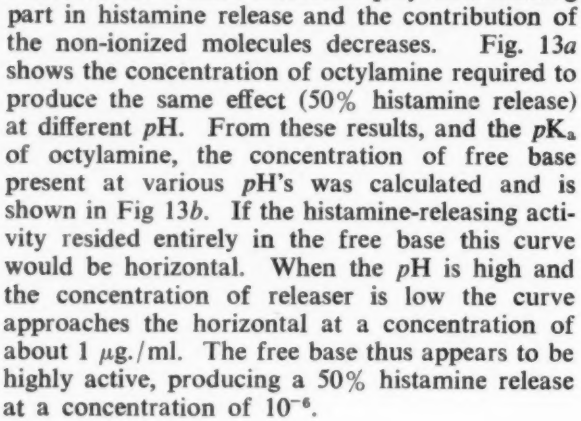


FIG. 13.—Analysis of effect of *p*H on the histamine-releasing activity of octylamine. In curve *a*, the concentrations of octylamine required to release 50% of the tissue histamine at 5 different *p*H have been plotted. In curve *b*, the corresponding concentrations of non-ionized base present at different *p*H have been calculated, based on a *p*K of 10.8.

When the *p*H is low and the concentration of releaser is high, the curve deviates from horizontal, indicating that the part played by the ionized base becomes increasingly important. This agrees with the finding that the fully ionized quaternary bases in high concentration are able to release histamine. Fig. 14 shows this for dodecyltrimethylammonium chloride (0.5 mg./ml.) and tubocurarine (1.7 mg./ml.). The activity of the quaternary compound

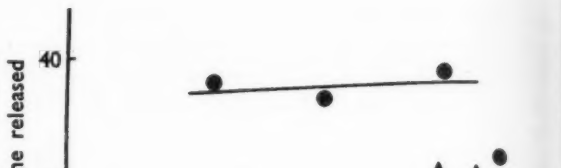


FIG. 14.—Effect of *p*H on histamine release from guinea-pig lung by quaternary amines. The release with C_{12} (0.5 mg./ml.) is depicted by the upper curve. The results with tubocurarine (1.7 mg./ml.), shown by the lower curve, were obtained from three separate experiments.

cannot be due to the presence of a small amount of the tertiary base, as the activity would not then be independent of *p*H. However, the most active quaternary compound is still 1,000 times less active as a histamine releaser from guinea-pig lung than the non-ionized octylamine molecule.

In investigating the effect of structure on histamine-releasing activity, a long-chain fatty acid (octanoic) and a weak base (aniline) were also studied. Both compounds were quite inactive when tested in relatively high concentrations (2 mg./ml. for octanoic and 1 mg./ml. for aniline).

Discussion

The effects of diamines fall into two groups: one type of effect has a maximum with a chain length of 4 or 5 carbon atoms. The other type increases steadily with chain length until it is limited by insolubility or micelle formation.

The short-chain compounds have three apparently related properties: they are substrates for histaminase; they inhibit histaminase competitively; and they potentiate the effects of histamine. It has been shown by Zeller (1942) that diamines of less than 4 carbon atoms are not as readily destroyed by diamine oxidase as those with 4 and 5 carbon atoms. Blaschko and Hawkins (1950) have shown that compounds with more than 5 carbon atoms become increasingly resistant to diamine oxidase. Our results on the inhibition of histaminase by diamines also show a maximum activity at C_4 or C_5, suggesting that inhibition of the destruction of histamine is due to a competition for the active enzyme centres.

The potentiation of the effects of histamine by cadaverine can be explained on similar lines. It has previously been shown that many inhibitors

of histaminase, including cadaverine, selectively potentiate the pharmacological effects of histamine, and that their effectiveness is correlated with their antihistaminase activity (Arunlakshana *et al.*, 1954). This interpretation of the potentiating effect of cadaverine is further supported by the finding that the long-chain diamines C_{10} and C_{15}, which produce little or no inhibition of histaminase, also fail to potentiate histamine.

It could be argued that cadaverine potentiates histamine not by inhibiting histaminase but by releasing histamine. It is, however, unlikely that histamine release can account for the potentiation, because the concentrations of cadaverine needed for histamine release are 1,000 times greater than those which potentiate histamine. It would also be expected that potentiation by histamine release would be non-specific. On the contrary, potentiation by cadaverine is specific and only occurs with compounds which are destroyed by histaminase (Arunlakshana *et al.*, 1954).

Each of the three effects of the short-chain compounds can thus be explained in terms of a specific fit of enzyme receptors requiring a critical distance between the polar groups on the molecule. The remaining three effects, histamine release, antagonism of histamine, and toxicity to paramecia, are also related in a common way to chain length: they occur only with the long-chain compounds and increase with chain length. The fact that there is a maximum with the monoamines is almost certainly due to the formation of micelles. An increase of activity with chain length is frequently found with compounds which exert a non-specific narcotic or toxic effect on cells, and can be explained by a decrease in solubility in the aqueous phase and a simultaneous increase in solubility in the lipoid layer of the cell. Non-ionized molecules are much more active than ionized molecules, as shown in Table IV, which gives the effective concentrations of the *active moiety* of various histamine releasers. They include unpublished results for pentamethonium and Schild's (1949) data for ammonia. The table shows the effect of ionization and chain length on histamine-releasing activity. Ammonia itself in the non-ionized form is a relatively weak releaser acting in a concentration of nearly 1 mg./ml. In the monoamine series the addition of a 4-carbon chain increases the activity 200-fold, and the addition of an 8-carbon chain 1,000-fold. Similarly, for the diamines, the 5-carbon chain is 50 times, and the 10-carbon chain 1,000 times, more active than ammonia. Both types of compound lose practically all their activity if ionized: all the quaternaries tested, including tubocurarine, have a very low activity, less than 1/1,000th of that of the non-ionized bases.

TABLE IV

COMPARISON OF INTRINSIC ACTIVITIES OF HISTAMINE RELEASERS

Compound	Carbon Atoms in Chain	Equi-active Total Conc. mg./ml.	pK_a	Active Moiety	Conc. of Active Moiety at *p*H 8·2 mg./ml.	Relative Activity
Primary monoamines: Ammonia	—	8·0	9·26	Non-ionized base	0·67	1
Butylamine	4	1·4	10·8	,,	0·0035	200
Octylamine	8	0·2	10·8	,,	0·0005	1,000
Primary diamines: Cadaverine	5	5·0	10·8	,,	0·013	50
Decamethylenediamine	10	0·2	10·8	,,	0·0005	1,000
Quaternary amines: Dodecatrimethylammonium	12	0·6	—	Ion	0·6	1·1
Pentamethonium (diquaternary)	5	12·0	—	,,	12·0	0·06

These results show that highly active aliphatic releasers possess the following properties: (i) They are basic molecules; for example, the monocarboxylic acid with an 8-carbon chain is completely inactive. (ii) They have a long hydrocarbon chain; ammonia is only weakly active. (iii) High activity is found mainly in the non-ionized form; the completely ionized quaternary compounds are only weakly active.

It is concluded from these results, obtained from simple but highly active molecules, that histamine release consists of two stages. The first is a non-specific uptake into the lipoid layer of the cell which involves the non-ionized form of the base; this conclusion is supported by the finding that the histamine releasers also have a non-specific depressant or narcotic action on cells as different as unicellular organisms and mammalian tissue cells. Once the base has entered the cell, the second stage of the release process takes place. This can be pictured as an ion exchange with histamine, since at the *p*H inside the cell both the releaser molecule and histamine are almost completely ionized.

In terms of this mechanism of action octylamine and decamethylene diamine are powerful histamine releasers, because (i) the non-ionized long-chain molecule can readily get into the cell and (ii) they are sufficiently strong bases to change back into the ionic form inside the cell. A very weak base such as aniline does not release hist-

amine, since although it fulfils the first requirement of lipoid solubility it is not sufficiently ionized to act as an ion exchanger inside the cell. The strong quaternary bases, on the other hand, fulfil the second requirement: they can act as ion-exchangers but they cannot readily get into the cell.

McIntire, Roth, and Sproull (1951) have proposed a different mechanism for histamine release by aliphatic monoamines from rabbits' blood. They found that the C_{18} primary amine released histamine and that its action could be antagonized by the C_{18} quaternary amine, which itself was inactive. This antagonism formed the basis for a "receptor" theory of histamine release with the primary and quaternary amines competing for the receptor sites. The findings can be interpreted as readily in terms of the proposed mechanism of non-specific uptake followed by ion exchange within the cell. The quaternary amine molecules are taken up by the cell by virtue of the lipoid solubility of their hydrocarbon chains, but, being fully ionized, are left with their "insoluble" charged heads still in the aqueous phase. While thus immobilized they would be able to antagonize histamine release by repelling the ionized releaser molecules.

References

Arunlakshana, O., Mongar, J. L., and Schild, H. O. (1954). *J. Physiol.*, **123**, 32.

Blaschko, H., Bülbring, E., and Chou, T. C. (1949). *Brit. J. Pharmacol.*, **4**, 29.

—— and Hawkins, J. (1950). Ibid., **5**, 625.

Hoerr, C. W., McCorkle, M. R., and Ralston, A. W. (1943). *J. Amer. chem. Soc.*, **65**, 328.

McIntire, F. C., Roth, L. W., and Sproull, M. (1951). *Amer. J. Physiol.*, **167**, 233.

MacIntosh, F. C., and Paton, W. D. M. (1949). *J. Physiol.*, **109**, 190.

Mongar, J. L., and Schild, H. O. (1953a). *Abstr. Commun. XIX int. physiol. Congr., Montreal*, p. 627.

—— —— (1953b). *Brit. J. Pharmacol.*, **8**, 103.

Ralston, A. W., and Hoerr, C. W. (1942). *J. Amer. chem. Soc.*, **64**, 772.

Rocha e Silva, M., and Schild, H. O. (1949). *J. Physiol.*, **109**, 448.

Schild, H. O. (1949). *Nature, Lond.*, **164**, 24.

Zeller, E. A. (1942). *Advanc. Enzymol.*, **2**, 93.

Brit. J. Pharmacol. (1957), **12**, 149.

A COMPARATIVE STUDY OF KININ, KALLIDIN, AND BRADYKININ

BY

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Partially purified kinin, a polypeptide in wasp venom, has been found to be a potent smooth-muscle stimulating and hypotensive agent. Such a preparation was 10 to 100 times more effective than histamine in enhancing capillary permeability on intradermal injection, and 10 times more effective than acetylcholine in evoking pain on a cutaneous blister base. Some differences between the actions of salivary kallikrein and trypsin in releasing kallidin or bradykinin have been observed, and some modifications of previous methods of preparing crude kallidin and bradykinin are suggested. Kallidin and bradykinin are effective enhancers of capillary permeability in the guinea-pig and rabbit. Chemical and pharmacological tests failed to differentiate between kallidin and bradykinin which must be, therefore, closely similar compounds. The possible role of kallidin and bradykinin in physiological or pathological conditions is discussed.

Kinin (Jaques and Schachter, 1954; Schachter and Thain, 1954), kallidin (Werle, Götze and Keppler, 1937; Werle and Berek, 1950; Werle, 1955) and bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949; Andrade, Diniz and Rocha e Silva, 1953; Rocha e Silva, 1955) are potent polypeptides, with many properties in common. All are known to produce a characteristic delayed slow contraction of the isolated guinea-pig ileum, and to lower markedly the arterial blood pressure of the rabbit and cat on intravenous injection. They are stable in neutral or acidic, but not in alkaline, solution; they are insoluble in anhydrous acetone or ether, and are rapidly inactivated by chymotrypsin. Kinin occurs in large quantities, together with histamine and 5-hydroxytryptamine, in the venom of the common wasp (*V. vulgaris*); kallidin and bradykinin are released from the α_2 globulin fraction of serum by the action of kallikrein and trypsin (or *B. jararaca* venom) respectively. In view of their similar properties it was of interest to extend previous studies of these three substances and to compare their properties under the same conditions. Preliminary results of such a comparison have been reported to the Physiological Society (Holdstock, Mathias and Schachter, 1956).

In the present experiments kinin has been further purified and characterized chemically and pharmacologically. It has been found to be extremely effective in enhancing capillary permeability and in evoking cutaneous pain on application to a blister base. Kallidin and bradykinin were also shown to increase capillary permeability. The different methods of preparing kallidin and bradykinin were compared, and some modifications of previous methods are described.

Despite their many common properties, kinin has been definitely distinguished from kallidin and bradykinin. The latter two substances, however, were not successfully distinguished by pharmacological or chemical tests, and must be closely similar, or possibly identical, compounds.

METHODS

Isolated Smooth Muscle and Arterial Blood Pressure.—Preparations of guinea-pig ileum, rabbit intestine, and rat uterus were suspended in Tyrode solution in an 18 ml. bath. Guinea-pig and rabbit intestine were kept at 34 to 36° C. and rat uterus (anoestrous) at 26 to 28° C., the latter temperature being required to eliminate spontaneous activity in this preparation. Atropine (0.2 μg.) and mepyramine (0.4 μg.) were added to the bath before each test, except when assaying for histamine, in which case mepyramine was omitted. Usually the polypeptide was in contact with the tissue for 60 sec.

Dogs were anaesthetized by intravenous injection of a mixture of chloralose (50 mg./kg.) and urethane (500 mg./kg.); rabbits received pentobarbitone sodium (30 mg./kg.) intravenously, supplemented by ether. The substances tested were injected into the femoral vein and the arterial blood pressure was recorded with a mercury manometer from a carotid artery.

Paper Chromatography. — Chromatograms, usually ascending, were run on Whatman No. 1 filter paper for 16 to 18 hr. at room temperature, in the N-butanol/acetic acid/water solvent described by Partridge (1948). Solutions were applied in narrow strips approximately 1 to 2 cm. long. Mixtures, such as kinin and bradykinin (or kallidin and bradykinin), were chromatographed after superimposing applications of the different substances, the solutions being allowed to dry between applications. After chromatography, the paper was dried in air and cut into parallel horizontal strips, strip 0 being 0.5 cm. on either side of the line of application, strips 1 to 10 measuring 1 cm. each, and the remaining strips, up to the solvent front, 2 cm. each. Each strip was eluted with 1.5 ml. Tyrode solution, and 0.2 ml. or more was tested on the isolated guinea-pig ileum or rat uterus. The amounts of kinin applied to a 2 cm. strip for chromatography varied from 25 to 100 units, and that of kallidin or bradykinin from 3 to 6 units.

Kinin was also chromatographed in ethyl methyl ketone/pyridine/water (60 : 15 : 25-homogeneous solvent) and in amyl alcohol/pyridine/water (35 : 35 : 30-organic layer).

Paper Electrophoresis.—This was carried out in the apparatus of Markham and Smith (1951) using electrolyte solutions of either N/1 acetic acid (*p*H 2.5) or M/20 sodium phosphate (*p*H 8.1) in the terminal compartments. The solution to be tested was applied as a narrow strip, 2 cm. long, to the middle of the filter paper (Whatman No. 1), which was immersed in carbon tetrachloride in the middle compartment. Electrophoresis was carried out for approximately 4.5 hr. at 220 V. D.C. The current in the experiments at *p*H 2.5 and at *p*H 8.1 was 0.3 to 0.5 mA and 1.8 to 3.0 mA, respectively. The paper was subsequently dried and cut into anodal and cathodal strips.

Capillary Permeability.—Guinea-pigs or rabbits were injected intravenously with a 5% solution of pontamine sky blue 6 BX (E. Gurr) in saline (1.2 ml./kg.), into the saphenous or marginal ear vein respectively, and the injection of the test substance was then made either intradermally (guinea-pig, 0.1 ml.; rabbit, 0.2 ml.) or intravenously. Details of this procedure have been described by Miles and Miles (1952).

Cutaneous Pain.—Superficial blisters (approximately 5 mm. diameter) were produced on the flexor surface of the forearm in three subjects, either by burning the skin with a heated spatula or by the application of a cantharidin plaster as described by Armstrong, Dry, Keele and Markham (1953). The test solutions (made up in Tyrode solution and kept at room temperature) were gently applied to the blister surface, left in contact for 1 to 2.5 min., and then washed off with Tyrode solution. Five minute intervals were allowed between washing off a solution and the next application. The intensity of the pain was arbitrarily graded by the subject into 5 "degrees." The subjective assessment of pain was recorded by a voltmeter modified by extending the pointer and increasing the torque of its movement by an elastic band, so that its pointer wrote on a smoked drum. The subject selected one of six of a series of push buttons (according to the absence or "degree" of pain) which switched in 0, 2, 4, 6, 8 or 10 V. derived from a low voltage source. The selection of any one push button automatically disconnected the previous circuit. The deflexion of the pointer increased linearly with the voltage.

Serum and Serum Globulin.—Ox blood was collected at slaughter, defibrinated, centrifuged at 3,000 rev./min. for 20 min. and the serum dialysed for 36 to 48 hr. at 4° C. (against 10 to 20 volumes of distilled water, changed 2 to 3 times). When heated serum was used, serum was heated for 3 hr. at 56 to 58° C. to destroy the kallidin- and bradykinin-inactivating peptidase (Werle, Götze and Keppler, 1937), centrifuged again, and then dialysed as described. It was then used immediately or frozen at −4° C. for future use.

Crude ox serum globulin was prepared from heated, dialysed serum (as described above) by addition of an equal volume of saturated ammonium sulphate, and precipitation was allowed to occur for about 1 hr. at room temperature. After centrifugation, the precipitate was dissolved in a volume of distilled water equal to one-half to two-thirds the original volume of serum. The globulin solution was then dialysed against running tap water overnight, at room temperature; the final volume of dialysed globulin solution was approximately equal to the original volume of serum. Globulin solution prepared in this way is referred to as "heated globulin."

Human Salivary Kallikrein.—Human mixed saliva was collected from normal individuals over a period of several hours and pooled. After centrifugation, the supernatant was slowly filtered with suction and the filtrate dialysed for 24 hr. (against 10 volumes distilled water changed 3 times) at 4° C. Four volumes of acetone (Analar) were then added slowly, and the mixture allowed to stand at room temperature for 20 min. for precipitation to occur. The material was again centrifuged, the supernatant solution discarded, and the residue dried over phosphorus pentoxide. This residue, which contained some water-insoluble material, possessed 100% of the kallikrein activity of the original saliva, as measured by its ability to release kallidin. The residue adhered firmly to the centrifuge tube as a thin coating and was not suitable for weighing, but, if dissolved in water and freeze-dried, it yielded a light, white, flocculent material, which was readily weighable. This method of preparing kallikrein differed slightly from that of Werle and Roden (1936), who precipitated it from saliva with 50% acetone. We increased the acetone concentration to 80%, since in our experience 50% acetone precipitated only a small fraction of the kallikrein in some instances; also, freeze-drying provided an excellent dry preparation.

Drugs and Other Materials.—Histamine was used as acid phosphate, mepyramine as maleate, and atropine as sulphate. Weights of histamine and mepyramine are expressed as base.

Trypsin and chymotrypsin were crystalline preparations (Armour) containing less than 50% $MgSO_4$. In later experiments, a salt-free preparation of crystalline trypsin was used.

A preparation of substance P from horse intestine was kindly provided by Professor J. H. Gaddum, and it contained 13.8 units/mg.

Units of Kinin, Kallidin and Bradykinin.—The unit of kinin or of kallidin was an arbitrarily selected weight of material (prepared according to the methods given below) which caused a strong contraction of the guinea-pig ileum. Such a contraction was produced by 1.0 units of kinin or 0.25 to 0.5 units of kallidin. The units are related to the weights of materials in the following sections.

Preparation of Kinin, Kallidin, and Bradykinin

Kinin.—Kinin was prepared from isolated, dried venom sacs (separated from the sting, duct, etc.) of the common wasp, *Vespa vulgaris*, as previously described (Schachter and Thain, 1954). Crude kinin preparations, which were used as standards, were prepared by extracting approximately 100 dried isolated venom sacs with 10 ml. 95% ethanol, 5 to 7 times. The residue, free of histamine and 5-hydroxytryptamine was dried and kept in a desiccator over P_2O_5. Different batches so prepared contained 1 unit of kinin in 5 to 20 μg. of the powder; such preparations are referred to as crude kinin. Some kinin was also extracted by washing with large amounts of ethanol, but most of the activity remained in the residue.

A more purified preparation of kinin was prepared by extracting 36 mg. venom sacs (approximately 100 sacs) with 2 ml. glacial acetic acid four times. The acetic acid extract was mixed with 15 volumes of anhydrous ether and kept at 4° C. for 2 hr., centrifuged, and the residue washed five times with 95% ethanol. The residue, which was practically invisible and spread over the centrifuge tube, was taken up in distilled water and freeze-dried, yielding 2.8 mg. of a white flocculent powder, which contained 1 unit of kinin in 0.4 μg. This preparation is very hygroscopic on exposure to air, but has retained its activity at room temperature in a desiccator for 9 months. Extraction and purification of kinin in this way resulted in recovery of 80 to 100% of the original activity. This preparation is referred to as purified, or partially purified, kinin.

Kallidin.—At first kallidin was prepared according to the method of Werle, Ehrlicher and Koebke (1951) with slight modifications. Heated, dialysed ox serum was incubated for 15 min. at 35° C. with a dry preparation of human salivary kallikrein (1 ml. serum to kallikrein equivalent of 1 ml. saliva) dissolved in a minimal volume of water. The reaction was terminated by the addition of 1/10 of the volume of N/1 H_2SO_4 to the mixture. This mixture was dialysed overnight against running distilled water. The total dialysate was approximately 20 times the volume of the dialysand and was neutralized by addition of saturated $Ba(OH)_2$, using a *p*H meter to determine the end point. The total dialysate was evaporated to a small volume under reduced pressure in a slow stream of nitrogen. The glass evaporator incorporated a water-cooled, double coil condenser. The dried residue (unsuitable for weighing) was dissolved in a small volume of distilled water, centrifuged to remove insoluble material, and freeze-dried, yielding a light, greyish, readily weighable powder. A powder prepared by the above procedure contained 1 unit of kallidin in 500 μg.; the total recovery of crude kallidin was 240 mg./100 ml. serum (4.8 units/ml. serum).

Kallidin was also prepared from serum by extraction with boiling ethyl alcohol as used in the preparation of bradykinin by Rocha e Silva *et al.* (1949). Heated, dialysed ox serum was incubated with kallikrein as described above, and the mixture added to twice its volume of boiling ethanol for 5 min. and then centrifuged. The supernatant solution was reduced to small volume in an evaporator as above, dried, and the residue ground to a fine homogeneous powder. This preparation was relatively inactive, containing only 1 unit in 10 mg. powder, but activity was increased to 535 μg./unit by extracting the powder with glacial acetic acid and precipitating the active material with 10 volumes of ether at 4° C. The final recovery of kallidin after these procedures was 3.4 units/ml. serum. The reason for the low activity of the boiling ethanol extract would appear to be that the precipitation of serum proteins by ethanol is greatly reduced in dialysed salt-free serum. Thus, boiling ethanol extraction of kallidin prepared in the same way, but from non-dialysed serum, yielded a preparation of 550 μg./unit without further purification, the recovery being 17.6 units/ml. serum. Extraction of kallidin with boiling ethanol yielded a suitable dry powder without freeze-drying.

The most effective and simple method of preparing crude kallidin was found to be the incubation of salivary kallikrein with " heated globulin " (see above) and subsequent extraction with boiling ethanol. " Heated globulin " solution was incubated for 15 min. at 35° C. with a dry preparation of human salivary kallikrein (dissolved in a minimal volume of H_2O); the concentration of kallikrein/ml. of globulin solution corresponded to that in 1 ml. pooled human saliva. The kallidin was then extracted with boiling ethanol and the alcoholic extract reduced in volume as above, and dried. This preparation contained 1 unit in 75 μg. of dry powder and the yield was 7.6 units/ml. serum. Purification of this preparation by extraction with glacial acetic acid and precipitation with ether produced more than a 3-fold increase in activity, namely 1 unit in 24 μg., but the recovery was only about 10%.

Bradykinin.—Bradykinin was prepared by the action of crystalline trypsin on ox serum globulin as described by Rocha e Silva *et al.* (1949), but with some modifications which increased the yield. Since the observations of Werle *et al.* (1937) indicated that the yield of kallidin from serum is increased if the kallidin-inactivating peptidase is destroyed by pre-heating the serum (we confirmed this observation), we compared the release of bradykinin by trypsin from heated and unheated serum, and from globulin prepared from heated or unheated serum. In all instances the concentration of kallidin reached a greater peak with the heated substrate, the greatest concentration occurring with " heated globulin."

We therefore employed " heated globulin " for the preparation of bradykinin as well as for the preparation of kallidin. Approximately 400 ml. of " heated globulin " solution (from 400 ml. dialysed ox serum) was incubated with salt-free crystalline trypsin (400 μg./ml.) for 20 min., the bradykinin extracted with boiling ethanol, and taken to dryness as described for kallidin. The total recovery of dry material was 1.8 g. which contained 1 unit of activity/140 μg.; the yield of bradykinin was 32 units/ml. This recovery is more than 50 times greater than that calculated from the figures of Rocha e Silva *et al.* (1949), and was probably due, in part, to the reduced inactivation of bradykinin by " heated globulin." Further purification by extraction with glacial acetic acid and precipitation with ether yielded a preparation containing 1 unit/100 μg., but the total recovery was only 51%.

Two preparations of bradykinin prepared by the action of *B. jararaca* venom on ox globulin were kindly provided by Dr. M. Rocha e Silva. They contained 1 unit in 100 μg., and 25 μg., respectively.

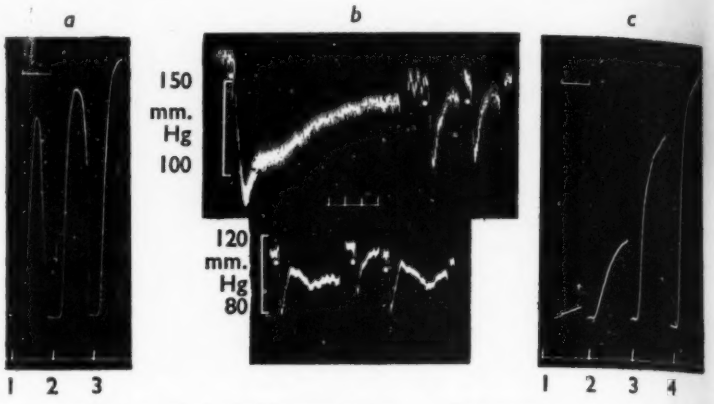

FIG. 1.—Effects of partially purified kinin (0.4 μg. = 1 unit) on different preparations. (*a*) Isolated rat uterus, 18 ml. bath, atropine (0.01 μg./ml.), 28° C. At 1, 2, and 3, addition of 0.1, 0.25, and 0.5 μg. kinin respectively. (*b*) Arterial blood pressure. Above: Dog, 15 kg., atropine (0.1 mg./kg.) and mepyramine (1.0 mg./kg.). At white dots, from left to right, 2.7, 0.7, and 0.7 μg./kg. kinin were administered intravenously. Below: Rabbit 2.8 kg., atropine (0.1 mg./kg.). At first and last dots, kinin, 1.0 μg./kg.; at middle dot, kallidin, 2 units/kg., were given intravenously. (*c*) Isolated guinea-pig ileum, atropine (0.01 μg./ml.) and mepyramine (0.02 μg./ml.), 35° C. At 1, 2, 3, and 4, addition of 0.1, 0.4, 0.7, and 1.0 μg. kinin respectively. Time, 30 sec.

RESULTS

Properties of Partially Purified Kinin

Effects on Smooth Muscle and Arterial Blood Pressure.—Kinin has previously been shown to contract smooth muscle and to lower the arterial blood pressure (Schachter and Thain, 1954). These properties have been confirmed, and the pharmacological studies extended with more purified material. The isolated rat uterus was the most sensitive preparation tested, but the guinea-pig ileum is the most suitable for assay, since a graded response is obtainable over a convenient range of concentrations. The order of sensitivity of the different preparations was: rat uterus > guinea-pig intestine > rabbit intestine. Rat uterus responded markedly to purified kinin in concentrations of less than 0.005 μg./ml. and the guinea-pig ileum to less than 0.025 μg./ml. (Fig. 1).

Purified kinin lowered the arterial blood pressure of the rabbit and dog on intravenous injection of 1.0 μg./kg., or less. This effect was undiminished on repeated injection and unaffected by treatment of the animal with atropine and mepyramine (Fig. 1). The hypotensive action of kinin, therefore, is not mediated through release of histamine. Also, on a weight basis, the purified preparation is far more effective than histamine in lowering the blood pressure of the rabbit or dog.

Cutaneous Pain.—Since some polypeptides have been shown to produce pain on application to a blister base on human skin (Armstrong, Keele, Jepson and Stewart, 1954), kinin was tested in similar experiments on 3 subjects. All subjects experienced moderate pain on application of concentrations of 100 μg./ml. of the purified preparation to a blister base; concentrations of 1 mg./ml. produced a more severe and protracted reaction. One subject, on whom lower concentrations were tested, reacted to solutions containing 20 μg./ml. but not to those with 10 μg./ml. Kinin was more effective than equal concentrations of acetylcholine in all subjects. The response differed from that to acetylcholine in that there was regularly a delay of 30 to 90 seconds before the subject responded; also, the pain reaction was generally more protracted than it was to acetylcholine (Fig. 2). The sensation of itchiness frequently occurred with the lower concentrations of kinin, or when the pain reaction decreased on prolonged contact with the solution.

Capillary Permeability.—The ability of kinin to increase capillary permeability to circulating dye was found to be one of its most striking pharmacological properties. Purified kinin was at least 10 times more effective than histamine in causing increased permeability to pontamine sky blue in the guinea-pig, and at least 100 times more effective

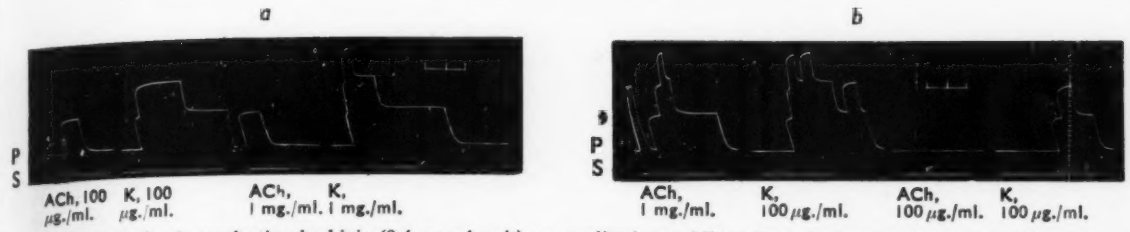

FIG. 2.—Records of pain production by kinin (0.4 μg. = 1 unit) on application to blister base on human skin. The pain response is recorded as an upward deflexion and is graded in arbitrary steps. (*a*) and (*b*) are experiments on two different subjects. P, pain response; S, stimulus (solution applied to blister base); K, kinin; ACh, acetylcholine. Time, 30 sec.

in similar experiments in the rabbit (Fig. 3). All animals blued effectively to concentrations of 0.1 μg./ml., and in some instances significant reactions were still detectable with less than 0.001 μg./ml. In both species the reaction generally appeared more quickly than did that to histamine, and, in general, the degree of blueness was greater than for histamine "lesions" of equal size.

On intravenous injection in the guinea-pig, kinin produced a generalized mild blueing reaction involving the entire skin, and also the visceral organs (Fig. 4). This differs from the regional blueing of the head and neck, presumably due to regional variations in histamine distribution in skin, caused by intravenous injections of the histamine liberator, 48/80 (Feldberg and Miles, 1953). The blueing of the internal organs of the guinea-pig produced by intravenous injections of purified kinin (5 to 25 μg./kg.) is in contrast to the negative or doubtful effects following the injection of equal amounts of histamine.

Paper Electrophoresis.—Crude preparations of kinin were found to migrate to the cathode, at approximately the same rate, whether at *p*H 2.6 or 8.1. In one experiment, approximately 200 units of crude kinin were electrophoresed (see Methods) using N/1 acetic acid (*p*H 2.6) as electrolyte. After 4 hr. of electrophoresis (0.3 to 0.5 mA) the paper was dried and cut into strips numbered 0 to 6 on each side of the line of application, and each strip eluted with 2 ml. saline. Strip 0 measured 0.5 cm. on either side of the line of application, and strips 1 to 6 were each 2.5 cm. wide. Large amounts of kinin were recovered in cathode strips 1 to 3, of which approximately 80% was in strip 2 (Fig. 5). No activity was detectable in the remaining eluates using guinea-pig ileum as test object.

Crude kinin was again similarly electrophoresed except that the electrolyte was M/20 sodium phosphate (*p*H 8.1). In this experiment the current varied from 2 to 3 mA and electrophoresis lasted 5 hr. The paper was dried and divided as before. Large amounts of kinin were recovered from the eluates of cathode strips 2 and 3 only, strip 2 having about twice the activity of strip 1. The anodal strips again possessed no pharmacological activity.

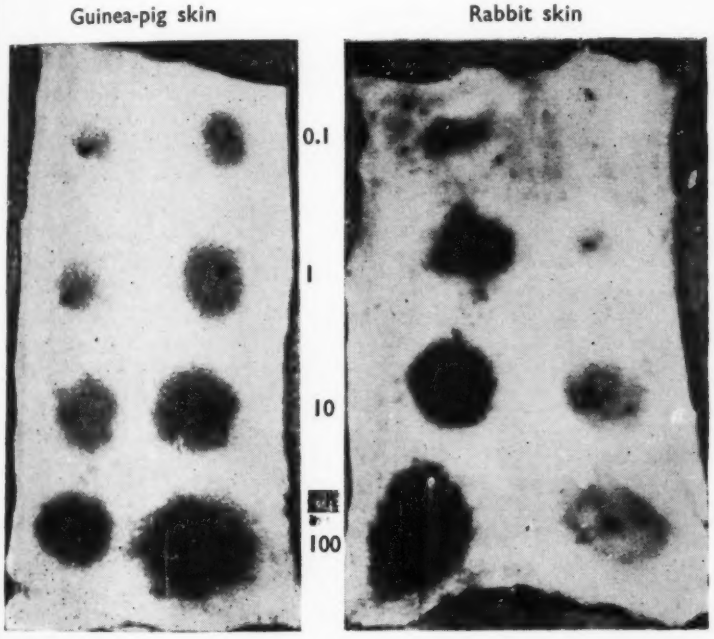

FIG. 3.—Effect of intradermal injections of kinin (0.4 μg. = 1 unit) and histamine on capillary permeability to a circulating dye in the guinea-pig and rabbit. The animals were previously injected with 5% pontamine blue (1.2 ml./kg.) intravenously. The dark areas are sites of intradermally injected kinin and histamine (volume: 0.1 ml. in the guinea-pig, 0.2 ml. in the rabbit) in concentrations of 0.1, 1, 10, and 100 μg./ml. from above downwards as indicated by the numerals between the two photographs. Note greater effectiveness of kinin.

FIG. 4.—Effect of intravenous injections of kinin (0.4 μg.=1 unit) and compound 48/80 on capillary permeability in the guinea-pig. All animals were previously injected with pontamine blue intravenously, and are depicted above 20 min. after the intravenous injection of: 1, kinin (50 units/kg.); 2, 48/80 (5 mg./kg.); 3, dye alone.

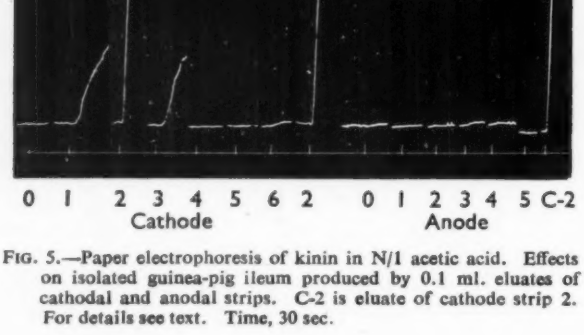

FIG. 5.—Paper electrophoresis of kinin in N/1 acetic acid. Effects on isolated guinea-pig ileum produced by 0.1 ml. eluates of cathodal and anodal strips. C-2 is eluate of cathode strip 2. For details see text. Time, 30 sec.

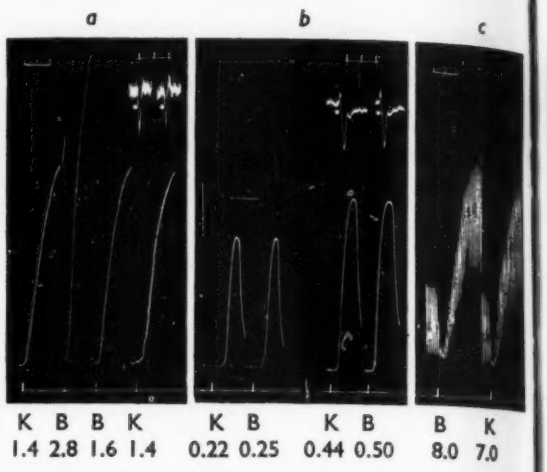

FIG. 6.—Comparison of the pharmacological activity of kallidin and bradykinin. K, kallidin; B, bradykinin. Numerals below K and B refer to units added to 18 ml. organ bath. Time, 30 sec. *a*, Isolated guinea-pig ileum, 35° C. Above: arterial blood pressure. Dog, 15 kg., chloralose-urethane. At white dots: left, 4.8 units bradykinin; right, 4.2 units kallidin. *b*, Isolated rat uterus, 28° C. Above: arterial blood pressure. Rabbit, 2.0 kg. Left, 4.8 units bradykinin; right, 4.2 units kallidin. *c*, Isolated rabbit jejunum, 35° C.

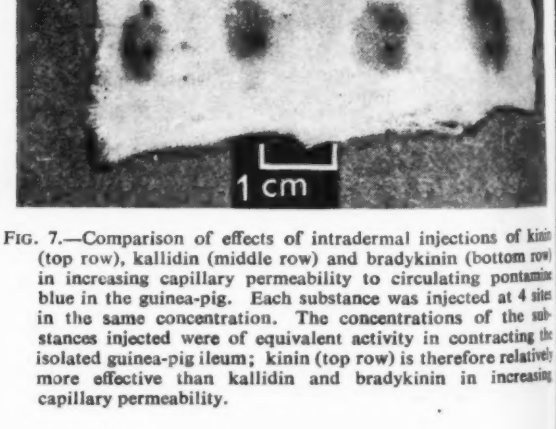

FIG. 7.—Comparison of effects of intradermal injections of kinin (top row), kallidin (middle row) and bradykinin (bottom row) in increasing capillary permeability to circulating pontamine blue in the guinea-pig. Each substance was injected at 4 sites in the same concentration. The concentrations of the substances injected were of equivalent activity in contracting the isolated guinea-pig ileum; kinin (top row) is therefore relatively more effective than kallidin and bradykinin in increasing capillary permeability.

Paper Chromatography.—Previous observations that crude kinin failed to move, or moved only slightly, from the application in ascending butanol-acetic acid chromatograms (Schachter and Thain, 1954) were confirmed. Furthermore, the same result was obtained with the most highly purified kinin in descending as well as ascending chromatograms in butanol-acetic acid, ketone-pyridine, or amyl alcohol-pyridine solvents.

Comparison of Kallidin and Bradykinin

Paper Chromatography.—Bradykinin and kallidin were first chromatographed separately in butanol-acetic acid solvent, and the eluates tested on the guinea-pig ileum. The R_F values of bradykinin in different experiments were 0.25, 0.26, 0.34, 0.35, and 0.37 respectively; those of kallidin were 0.28, 0.30, 0.32, and 0.35. In all instances, elution of the chromatogram yielded only a single pharmacologically active " peak " which was fairly well defined, occupying 2.5 cm. or less of a horizontal strip. In four of these experiments the bradykinin preparation was one prepared by the action of *B. jararaca* venom on globulin; in the experiment yielding an R_F value of 0.35 the bradykinin was prepared by the action of trypsin on " heated globulin." The kallidin preparations were those prepared by the action of human salivary kallikrein on heated serum, and subsequent recovery of kallidin in the dialysate (see Methods).

Similar experiments were also carried out but with the applications of bradykinin (prepared as above with either venom or trypsin) and kallidin (prepared as above) superimposed on each other on the paper and chromatographed. In all instances the pharmacological activity was again eluted as a single " peak." The R_F values of the mixture of kallidin and bradykinin did not differ significantly from those in which they were chromatographed singly.

Paper Electrophoresis.—Kallidin (heated serum+kallikrein, dialysis) and bradykinin (globulin+*B. jararaca* venom) were subjected to paper electrophoresis for 4 hr. at 0.3 to 0.5 mA in N/1 acetic acid as electrolyte. Since both substances migrated to the cathode at similar rates, attempts at separation by this method were not pursued. In both instances activity was found in the cathode strip approximately 3 cm. from the application. The anodal eluates were inactive when tested on the rat uterus or guinea-pig ileum.

Parallel Assays.—Bradykinin (globulin+*B. jararaca* venom) was assayed against kallidin (heated ox serum+kallikrein, dialysis) on 5 different test preparations. Their activities were qualitatively and quantitatively parallel in all instances. Assayed on the guinea-pig ileum 1 unit of kallidin was equivalent to 1.1 units of bradykinin. The same ratio of activity was found for the rat uterus, rabbit intestine, and rabbit or dog arterial blood pressure (Fig. 6). A similar comparison was made of their effects on capillary permeability in the guinea-pig, using kallidin which was prepared somewhat differently (heated globulin+kallikrein, ethanol extraction). Again, they were indistinguishable in their ability to increase capillary permeability to a circulating dye. However, concentrations of kinin which were equivalent to those of kallidin and bradykinin in contracting the guinea-pig ileum were considerably more effective in increasing capillary permeability in this species (Fig. 7).

Specific Desensitization.—As we observed with kinin, the guinea-pig ileum could not be desensitized by prolonged contact with high concentrations of kallidin or bradykinin. Pharmacological distinction of these polypeptides by this method is therefore impossible.

Proteolytic Enzymes.—Incubation of kallidin or bradykinin (0.5 to 1.0 unit/ml.) with an equal volume of human serum or of crystalline chymotrypsin (100 μg./ml.) for 20 min. at 35° C. resulted in the loss of 80 to 100% of activity. Activity was unaffected by incubation with crystalline trypsin under the same conditions. Similar results were obtained with the different preparations of kallidin and bradykinin.

Distinction of Kinin from Kallidin and Bradykinin

Paper Chromatography.—A distinct separation of kinin from mixtures containing kallidin or bradykinin was regularly and easily obtained by chromatography in butanol-acetic acid solvent. Kinin was regularly eluted from the origin of the chromatogram, whereas the others were obtained from a strip with an R_F of approximately 0.35. The result of a typical experiment is shown in Fig. 8. Kinin was readily distinguished from substance P in the same way, since the latter was recovered from the paper at an R_F of 0.32, which agrees with the value obtained by Pernow (1953).

Proteolytic Enzymes.—Kinin differs from kallidin or bradykinin in its greater susceptibility to inactivation by crystalline trypsin. Thus, the incubation of kinin (1 unit/ml.), irrespective of its state of purity, with crystalline trypsin (50 μg./ml.) for 20 min. at 35° C., resulted in the loss of 70 to 100% of its activity.

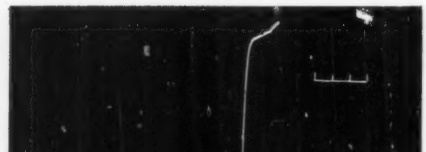

FIG. 8.—Contractions of isolated guinea-pig ileum showing separation of kinin from bradykinin by paper chromatography in butanol-acetic acid solvent. Atropine (0.01 μg./ml.) and mepyramine (0.02 μg./ml.) were present in the 18 ml. bath, 35° C. Time, 30 sec. 0 to 8 are the responses to eluates of horizontal chromatogram strips after running a mixture of kinin and bradykinin as described in the text.

Rabbit Blood Pressure.—A qualitative difference between the effects of kinin and kallidin or bradykinin was the distinct secondary depression of the arterial blood pressure consistently seen only with kinin (Fig. 1*b*). This characteristic has been previously noted with cruder preparations of kinin (Schachter and Thain, 1954).

Comparison of the Release of Kallidin and Bradykinin

Both human salivary kallikrein and bovine pancreatic trypsin released higher concentrations of kallidin and bradykinin, respectively, from serum or globulin which had been previously heated. With heated serum or globulin, the concentration of the smooth muscle stimulant increased for about 20 min. of incubation, and then decreased slowly, so that high concentrations were still present even after 1 hr. With unheated substrates, however, the concentration of kallidin or of bradykinin decreased considerably after 5 to 10 min. of incubation. The more effective release from heated substrates was presumably due to the inactivation of serum peptidase by mild heating (56° C. for 2 to 3 hr.), which does not affect kallidinogen or bradykininogen (Werle *et al.*, 1937). Fig. 9 shows the greater concentrations of bradykinin obtained by the action of trypsin on heated, than on unheated, globulin.

It was also observed that the optimal conditions for the release of kallidin were not identical with those for the release of bradykinin. Thus, the ratio of concentrations of kallidin after 20 min. incubation at 35° C. of salivary kallikrein with heated serum and "heated globulin," respectively, was approximately 1.5/1; with trypsin, however, under the same conditions, the ratio of bradykinin concentrations was approximately 1/4. This reversal of the ratio suggests a difference between the actions of kallikrein and trypsin, the exact nature of which requires further analysis.

Since kallikrein has not been obtained in a pure state, it is not possible to compare quantitatively its effectiveness in releasing kallidin with that of trypsin in releasing bradykinin. However, kallikrein must be a far more potent agent than trypsin in this respect, since incubation of a very crude preparation of human salivary kallikrein, in a concentration of less than 1.0 μg./ml., produced large amounts of kallidin from 0.5 ml. heated ox serum. Crude kallikrein was at least 10 times as effective as crystalline trypsin in this respect.

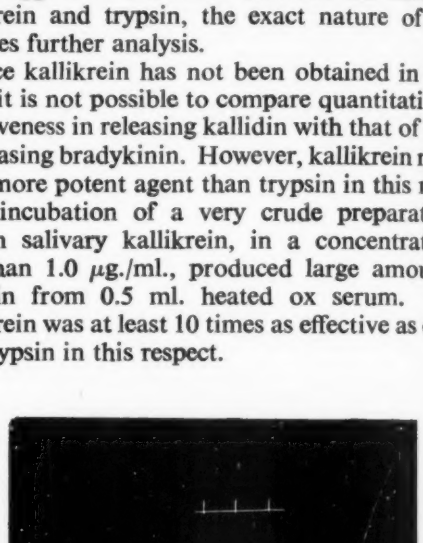

FIG. 9.—Contractions of isolated guinea-pig ileum in response to bradykinin released by crystalline trypsin from unheated and "heated globulin." 1 and 2: 0.5 ml. globulin solution + 0.1 ml. trypsin (1 mg./ml.) incubated for 2 and 2½ min. respectively. 1′ and 2′: as in 1 and 2 but using "heated globulin." 18 ml. bath, atropine (0.01 μg./ml.) and mepyramine (0.02 μg./ml.), 35° C. Time, 30 sec.

Discussion

The present experiments confirm previous observations indicating the pharmacological similarity of kinin, kallidin and bradykinin. It has, however, proved possible to distinguish kinin from these other compounds by paper chromatography and by differential sensitivity to inactivation by trypsin. Since kallidin and bradykinin were indistinguishable by all the comparative tests, they must be closely similar compounds. None the less, our experiments cannot be regarded as proving them to be identical, since it is possible for polypeptides to be indistinguishable in their pharmacological and chemical properties and yet differ in their chemical composition. Thus, pure vasopressins of bovine and porcine origin differ only in that the former contains lysine, and the latter arginine, as its basic amino acid (du Vigneaud, Lawler and Popenoe, 1953). A final answer regarding the distinction of bradykinin from kallidin must, therefore, await studies on pure preparations.

A number of the observations *in vitro* suggests the possibility that the activation of serum kallikreinogen, or the release of kallidin, might occur *in vivo*. For example, serum kallikreinogen is activated *in vitro* by changes in *p*H (Werle, 1934), by papain (Kraut, Frey and Werle, 1933), and by trypsin (Werle, Forell and Maier, 1955); also, substances resembling kallidin and bradykinin are released when serum comes into contact with glass (Armstrong, Keele, Jepson and Stewart, 1954) or when it is diluted (Schachter, 1956). On the basis of the activation *in vitro* of kallikreinogen by *p*H changes, experiments were carried out on reactive hyperaemia by Frey (1930), who obtained indirect evidence suggesting that serum kallikreinogen was activated during circulatory arrest and accounted for the subsequent hyperaemia. However, the release of kallidin into the venous outflow of the dog's hind limb could not be demonstrated after 10 min. of arrest of the arterial supply (Schachter, unpublished).

Ungar and Parrot (1936) suggested that the vasodilatation of the submaxillary gland and tongue during stimulation of the lingual nerve was due to the secretion of salivary kallikrein into the blood circulation. Hilton and Lewis (1955a and b) have recently demonstrated that a " bradykinin-releasing enzyme " appears in the effluent of the Tyrode-perfused submaxillary gland during stimulation of the chorda tympani nerve, and have suggested that vasodilatation in the salivary gland is due to this " enzyme " being secreted into the capillaries of the tissue during salivary secretion. There is no evidence that the " bradykinin-releasing enzyme " in saliva described by these authors differs from salivary kallikrein. Their observations do, however, support the possibility that such a mechanism operates to produce local vasodilatation in the secreting salivary gland. The physiological role of pancreatic kallikrein remains completely obscure, since it exists in the pancreas in an inactive form (Werle, 1937), and, even if it were secreted into the blood stream, it would be pharmacologically inert. On the other hand, it is activated in the duodenum by trypsin (Werle and Urhahn, 1940), but there it has no known digestive or other function. Pancreatic trypsin also exists in an inactive form in the pancreas, and it is similarly difficult to envisage how its possible slow release into the blood circulation during pancreatic activity could result in the release of bradykinin.

The ability of these polypeptides to evoke pain, to enhance permeability, and to cause vasodilatation would make them extremely effective mediators of inflammatory reactions, but definite evidence of their contribution to any type of injury reaction has not yet been presented. Therefore, despite the pharmacological potency of kallidin and bradykinin, and despite the fact that endogenous mechanisms exist for their potential elaboration in high concentrations, their role in physiological or pathological processes remains obscure.

Wasp venom is able to release histamine, and also contains high concentrations of histamine, 5-hydroxytryptamine, kinin (Jaques and Schachter, 1954; Schachter and Thain, 1954) and hyaluronidase (Jaques, 1955). Since the present experiments demonstrate the potent pain-producing and capillary permeability enhancing properties of kinin, there remains little, if anything, to be accounted for in the local reactions to a wasp sting. It does not follow, however, that the occasional severe generalized reaction of humans to a wasp sting (Brown, 1944) is due to these agents in the venom. Although it is possible that the intravenous injection of the contents of one wasp gland would produce a reaction of this nature in any individual, it is also possible that these rare reactions are truly anaphylactic, that is, due to previous sensitization to venom protein.

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References

Andrade, S. O., Diniz, C. R., and Rocha e Silva, M. (1953). *Arch. int. Pharmacodyn.*, **45**, 100.

Armstrong, D., Dry, R. M. L., Keele, C. A., and Markham, J. W. (1953). *J. Physiol.*, **120**, 326.

—— Keele, C. A., Jepson, J. B., and Stewart, J. W. (1954). *Nature, Lond.*, **174**, 791.

Brown, E. A. (1944). *Ann. Allergy*, **2**, 235.

Feldberg, W., and Miles, A. A. (1953). *J. Physiol.*, **120**, 205.

Frey, E. K. (1930). *Arch. f. klin. Chirurg.*, **162**, 334.

Hilton, S. M., and Lewis, G. P. (1955a). *J. Physiol.*, **128**, 235.

—— —— (1955b). Ibid., **129**, 253.

Holdstock, D. J., Mathias, A. P., and Schachter, M. (1956). Ibid., **133**, 14P.

Jaques, R. (1955). *Helv. physiol. Acta*, **13**, 113.

—— and Schachter, M. (1954). *Brit. J. Pharmacol.*, **9**, 53.

Kraut, H., Frey, E. K., and Werle, E. (1933). *Hoppe-Seyl. Z.*, **222**, 73.

Markham, R., and Smith, J. D. (1951). *Nature, Lond.*, **168**, 406.

Miles, A. A., and Miles, E. M. (1952). *J. Physiol.*, **118**, 228.

Partridge, S. M. (1948). *Biochem. J.*, **42**, 238.

Pernow, B. (1953). *Acta physiol. scand.*, **29**, Suppl. 105.

Rocha e Silva, M., Beraldo, W. T., and Rosenfeld, G. (1949). *Amer. J. Physiol.*, **156**, 261.

—— (1955). In *Polypeptides which Stimulate Plain Muscle* (J. H. Gaddum, ed.). Edinburgh: Livingstone.

Schachter, M., and Thain, E. M. (1954). *Brit. J. Pharmacol.*, **9**, 352.

—— (1956). Ibid., **11**, 111.

Ungar, G., and Parrot, J. L. (1936). *C.R. Soc. Biol., Paris*, **122**, 1052.

du Vigneaud, V., Lawler, H. C., and Popenoe, E. A. (1953). *J. Amer. chem. Soc.*, **75**, 4880.

Werle, E. (1934). *Biochem. Z.*, **273**, 291.

—— (1937). Ibid., **290**, 129.

—— (1955). In *Polypeptides which Stimulate Plain Muscle* (J. H. Gaddum, ed.). Edinburgh: Livingstone.

—— and Berek, U. (1950). *Biochem. Z.*, **320**, 136.

—— Ehrlicher, R., and Koebke, K. (1951). Ibid., **322**, 27.

—— Forell, M. M., and Maier, L. (1955). *Arch. exp. Path. Pharmak.*, **225**, 369.

—— Götze, W., and Keppler, A. (1937). *Biochem. Z.*, **289**, 217.

—— and Roden, P. V. (1936). Ibid., **286**, 213.

—— and Urhahn, K. (1940). Ibid., **304**, 387.

Brit. J. Pharmacol. (1957), **12**, 159.

THE RELATIONSHIP BETWEEN INHIBITION OF PHOSPHOFRUCTOKINASE ACTIVITY AND THE MODE OF ACTION OF TRIVALENT ORGANIC ANTIMONIALS ON *SCHISTOSOMA MANSONI*

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The addition of purified mammalian phosphofructokinase to homogenates of *Schistosoma mansoni* increased the rate of lactic acid production from glucose and reversed the inhibition of glycolysis produced by low concentrations of trivalent organic antimonials. Neither mammalian phosphofructokinase nor trivalent antimonials affected the rate of lactic acid production from fructose-1 : 6-diphosphate (HDP) by schistosome homogenates. Accordingly, in the schistosome, the rate of glycolysis of glucose is determined by the activity of phosphofructokinase.

The aldolase of *S. mansoni* has a high requirement for HDP; relatively slight reductions in the concentration of this substrate below the optimum resulted in a sharp decline of aldolase activity. Therefore, decreased formation of HDP, due to inhibition of schistosome phosphofructokinase activity by antimonials, reduced the activity of aldolase and resulted in an inhibition of glycolysis of schistosome homogenates.

Kinetic data revealed differences in the nature of the phosphofructokinase of *S. mansoni* and that of the enzyme catalysing the same reaction in the host. Exposure of schistosomes to low concentrations of potassium antimonyl tartrate or administration of subcurative doses of stibophen to the host resulted in an accumulation of the substrate (fructose-6-phosphate), and a reduction of the product (HDP) of the phosphofructokinase reaction, indicating that the activity of this enzyme was inhibited by antimonials in the intact parasite. It is concluded that inhibition of phosphofructokinase activity can account for the mechanism of the chemotherapeutic action of trivalent organic antimonials in schistosomiasis.

Despite their relatively high toxicity and other undesirable properties, trivalent organic antimonials are still considered to be the most effective available chemotherapeutic agents in the treatment of schistosomiasis. Information about the mechanism of the schistosomicidal action of these drugs might reveal opportunities for the development of compounds equally effective against the parasite, but less damaging to the host. In an attempt to explore this problem it was observed that trivalent antimonials produce a decrease in the utilization of carbohydrate by *Schistosoma mansoni* (Bueding, 1950). Subsequent studies revealed that organic antimonials selectively inhibit the phosphorylation of fructose-6-phosphate by adenosine triphosphate, catalysed by the enzyme phosphofructokinase (Mansour and Bueding, 1954). The present paper is concerned with the relationship between this biochemical effect of trivalent antimonials and their mode of action in schistosomiasis. The reaction sequences which are discussed are shown in Fig. 1.

METHODS

In this paper the following abbreviations are used: F-6-P, Fructose-6-phosphate; HDP, fructose-1 : 6-diphosphate; G-6-P, glucose-6-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

The preparation of schistosome homogenates and measurement of their rate of glycolysis were carried out as in a previous study (Mansour and Bueding, 1954). Phosphofructokinase of rabbit muscle was prepared according to Ling, Byrne, and Lardy (1955), aldolase according to Taylor, Green, and Cori (1948),

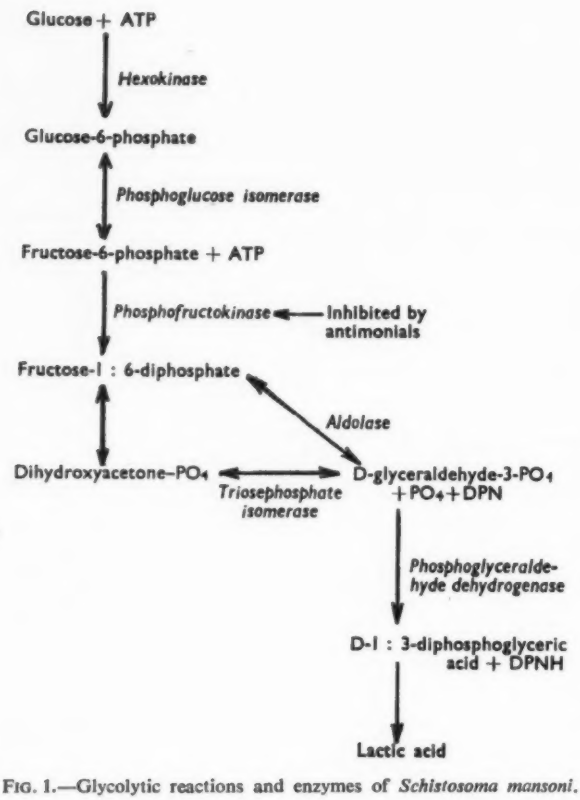

FIG. 1.—Glycolytic reactions and enzymes of *Schistosoma mansoni*.

and glyceraldehyde phosphate dehydrogenase according to Cori, Slein, and Cori (1948). Glucose-6-phosphate dehydrogenase from yeast was prepared by a procedure identical to that used in a previous study (Bueding and MacKinnon, 1955a). An extract containing schistosome phosphofructokinase was obtained as follows: adult schistosomes were cut with a pair of small scissors in potassium glycylglycine buffer (*p*H 7.5; 0.01 M; 50 worm pairs/ml.) for 2 min. After stirring for another 2 min. the mixture was centrifuged at 3,500 *g* and the supernatant was used as the source of the enzyme. All operations were carried out between 0 and 4° C.

Enzymic activities are expressed in arbitrary units. One unit is defined as the amount of enzyme which turns over 1 μM of substrate in 1 min. at 25° C. Rates of phosphofructokinase activity were determined spectrophotometrically according to Ling *et al.* (1955) (*System b*). In the presence of excesses of aldolase and of glyceraldehyde-3-phosphate dehydrogenase, the formation of HDP, catalysed by phosphofructokinase, was followed by the conversion of HDP to glyceraldehyde-3-phosphate and the oxidation of the latter by DPN. Reduction of DPN was measured in a Beckman spectrophotometer at 340 mμ. It was possible to use this procedure because schistosome homogenates and extracts did not contain any measurable 2-glycerophosphate dehydrogenase activity.

The concentration of hexose phosphates in the worm was determined in the following manner: the parasites were homogenized in 0.2 N-HCl (1 ml./100 worm pairs) in an all-glass homogenizer. The homogenate was centrifuged for 20 min. at 34,000 *g* in a Spinco preparative ultracentrifuge. The supernatant was neutralized with 0.16 volumes of 2 N-$KHCO_3$ and then centrifuged for 20 min. at 34,000 *g*. Aliquots of the second supernatant (S2) were assayed for HDP, F-6-P, and G-6-P as described below.

Determination of HDP.—Glyceraldehyde phosphate dehydrogenase (0.8 units) was added to a Beckman spectrophotometer cell (capacity, 1 ml.; light path, 10 mm.) containing an aliquot of S2, potassium glycylglycine buffer (*p*H 8.3; 5×10^{-2} M), $MgCl_2$ (5×10^{-3} M), potassium arsenate (6×10^{-3} M), L-cysteine (1×10^{-3} M), and neutralized DPN (3.4×10^{-4} M), to give a total volume of 0.8 ml. No reduction of DPN was observed at a wavelength of 340 mμ. Therefore, no triose phosphate was detectable in S2. Aldolase (0.5 units) was then added and the subsequent reduction of DPN was equivalent to the molar amount of HDP present in S2. Reduction of DPN was completed within 15 min.

Determination of F-6-P and G-6-P.—0.05 units of glucose phosphate dehydrogenase was added to a spectrophotometer cell containing an aliquot of S2, potassium glycylglycine buffer (*p*H: 8.3; 5×10^{-2} M), $MgCl_2$ (1.25×10^{-2} M), and TPN (1.5×10^{-4} M). The final volume was 0.8 ml. The G-6-P present in S2 was completely oxidized in 15 min. with a corresponding reduction of TPN. The enzyme preparation was contaminated with a small amount of phosphohexose isomerase, and therefore 20 to 30% of the F-6-P in S2 was converted to G-6-P. This percentage was determined by assaying a standard solution of F-6-P along with each unknown. After 15 min., an excess of rabbit muscle phosphohexose isomerase was added and the subsequent reduction of TPN was proportional to the F-6-P remaining in S2. The total F-6-P in S2 was calculated from the amount of TPN reduced after addition of phosphohexose isomerase, plus % of the standard F-6-P solution which was converted to G-6-P in the first 15 min. The amount of G-6-P present in S2 was calculated by subtracting the value found for F-6-P from the total hexosephosphates. Calculations of the amounts of reduced DPN and TPN were based on their molar extinction coefficients of 6.3×10^3 at the wavelength of 340 mμ.

In each of the experiments described below worms were used which originated from mice infected on the same day by the same group of cercariae; in these worms variations in the respective concentrations of the three phosphate esters did not exceed 15%. Much greater differences were encountered among schistosomes removed from animals infected with different batches of cercariae.

Aldolase and glyceraldehyde phosphate dehydrogenase activities of schistosomes were measured spectrophotometrically for a period of 5 min. with

predetermined optimal concentrations of the various constituents of the reaction mixture. These are given below; for aldolase: potassium glycylglycine buffer: *p*H 8.3, 5×10^{-2} M; MgHDP: 5×10^{-3} M; potassium arsenate: 6×10^{-3} M; DPN: 5×10^{-4} M; glyceraldehyde phosphate dehydrogenase: 0.8 units/ml.; for glyceraldehyde phosphate dehydrogenase: potassium glycylglycine buffer: *p*H 8.7; 5×10^{-2} M; L-cysteine: 1×10^{-3} M; DPN 1.4×10^{-3} M; potassium arsenate: 6×10^{-3} M; MgHDP: 5×10^{-3} M; aldolase: 0.5 units per ml.

Lactic dehydrogenase activity of *S. mansoni* was determined according to a procedure described previously (Mansour and Bueding, 1953).

The commercially available magnesium salt of HDP was purified by dissolving 100 mg. of this material in 1 ml. of ice-cold water and adding 30 mg. of charcoal (Nuchar). After stirring the mixture for 3 min. it was filtered and the colourless filtrate was used.

Results

Addition of purified mammalian phosphofructokinase resulted in an increase in the rate of glycolysis of schistosome homogenates when glucose was used as the substrate (Table I). These observations indicated that the rate of the reaction catalysed by phosphofructokinase determined the rate of lactic acid production from glucose by cell-free extracts of the worms. Other evidence recorded in Table I suggests that inhibition of

TABLE I

LACTIC ACID PRODUCTION BY HOMOGENATES OF *SCHISTOSOMA MANSONI*

Substrates: Glucose+ATP. Phosphofructokinase (PFK) from rabbit muscle (0.5 to 0.6 units/ml.). Activities are expressed as μ moles of lactic acid produced/mg. protein in 30 min. (37° C.).

Additions	Activity			
	1	2	3	4
None	1·4	1·1	0·5	1·2
Stibophen (1×10^{-4} M)	0·6	0·2	0·2	0·4
PFK	2·1	1·4	1·8	2·0
Stibophen+PFK	2·3	1·5	1·9	1·9
Potassium antimonyl tartrate (5×10^{-5} M)				0·4
Potassium antimonyl tartrate +PFK				1·7

phosphofructokinase activity by trivalent antimonials can account for the inhibition of glycolysis. For example, the inhibitory effects of stibophen and of potassium antimonyl tartrate on lactic acid production from glucose were abolished by the addition of phosphofructokinase of rabbit muscle. Neither phosphofructokinase nor the antimonials altered the rate of lactic acid production when fructose-1:6-diphosphate instead of glucose and ATP was used as substrate (Table II).

TABLE II

LACTIC ACID PRODUCTION BY HOMOGENATES OF *SCHISTOSOMA MANSONI*

Substrate: Hexose diphosphate. Activities and PFK as in Table I.

Additions	Activity			
	1	2	3	4
None	1·1	1·7	1·9	1·9
PFK	1·1	1·7	2·0	1·9
Stibophen (1×10^{-4} M)	1·1		2·1	1·7
Potassium antimonyl tartrate (7×10^{-5} M)			1·9	1·7

The experiments described above suggested that the phosphofructokinase reaction might be the rate-limiting process in the glycolysis of schistosomes; that is, the conversion of F-6-P to HDP might proceed at a slower rate than any other step in the series of reactions concerned with the production of lactic acid from glucose. Accordingly, the reaction rates of five glycolytic enzymes of schistosomes were measured under optimal conditions, which did not differ substantially from the standard conditions used in the earlier experiments. The activities of the enzymes, expressed as μ moles of substrate utilized/mg. of protein/min. at 25° C., were as follows: Phosphohexose isomerase, 0.42; phosphofructokinase, 0.42; aldolase, 0.17; phosphoglyceraldehyde dehydrogenase, 1.58; and lactic dehydrogenase, 0.36. The rate of the reaction catalysed by the phosphofructokinase of the parasite was considerably more rapid than the next step in glycolysis, namely the formation of two molecules of triosephosphate from one molecule of HDP catalysed by aldolase. Therefore, the rate of the phosphofructokinase reaction was not limiting the rate of glycolysis of schistosomes. The possibility was then explored that the concentration of HDP, the product of the phosphofructokinase reaction, might affect the rate of glycolysis of the parasite. Fig. 2 shows the effect of the concentration of HDP on the activity of aldolase. Optimal activity was observed at a relatively high substrate concentration (5×10^{-3} M). If the concentration of the substrate was reduced below a certain critical level (1×10^{-3} M) there was a very sharp decline in the activity of aldolase.

This low activity was not caused by instability of the enzyme in the presence of sub-optimal concentrations of substrate. In several experiments aldolase activity was measured initially with HDP at a concentration of 3×10^{-4} M for 5 min.; when subsequently the substrate concentration in the reaction mixture was raised to the optimum (5×10^{-3} M), the enzymic activity was as high as in the

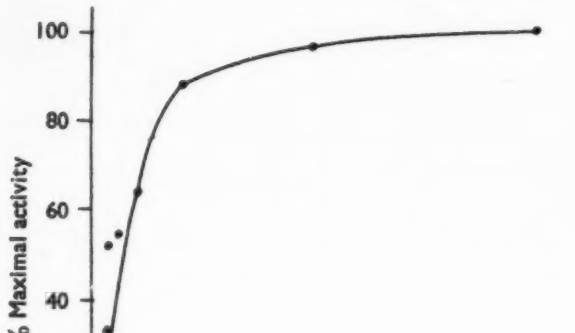

FIG. 2.—Relation of substrate concentration to the activity of aldolase of *Schistosoma mansoni*.

control assay in which aldolase activity was determined at once at the optimal substrate concentration without prior exposure of the enzyme to a lower concentration of HDP. Since the concentration of HDP in schistosomes never exceeded 3×10^{-4} M (see below), a slight decrease below this concentration, due to inhibition of phosphofructokinase activity by antimonials, would markedly reduce the activity of aldolase, resulting in an inhibition of glycolysis. Conversely, addition of mammalian phosphofructokinase to homogenates of schistosomes would increase the rate of HDP formation, giving rise to a significant increase in aldolase activity, thereby increasing the rate of lactic acid production. Furthermore, inhibition of HDP formation by antimonials should be abolished by the addition of an excess of mammalian phosphofructokinase to schistosome homogenates. These are the changes that were found experimentally (Table I).

The observations reported above were made with cell-free preparations of the worms; the problem arose whether similar mechanisms operate in the intact parasite. It was found in earlier studies that utilization of glucose and production of lactic acid by intact schistosomes are reduced by antimonials in concentrations similar to those which inhibited the activity of the parasite's phosphofructokinase (Bueding, 1950). If antimonials were inhibiting this enzyme in intact worms, they would produce an increase in the concentration of the substrate and a decrease in the concentration of the product of the phosphofructokinase reaction. Accordingly, schistosomes were incubated in dilute horse serum (3 vols. serum + 1 vol. water) containing potassium antimonyl tartrate in a concentration (5×10^{-5} M) which markedly inhibits the activity of schistosome phosphofructokinase and the rate of glycolysis of worm extracts. Exposure to the same concentration of the antimonial reduced the survival of the worms *in vitro* from 30 days to approximately 8 hr. The concentrations of hexosemonophosphates and of HDP were determined as soon as a definite reduction in motility became evident;

TABLE III

EFFECT OF POTASSIUM ANTIMONYL TARTRATE ON THE CONCENTRATIONS OF HEXOSEPHOSPHATES IN SCHISTOSOMES

The period of incubation in 75% horse serum at 37° C. was 1 hr. in experiments 1 to 5 and 2 hr. in experiment 6.

Expt.	Molar Conc. of Antimonial	F-6-P		G-6-P		HDP	
		Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change
1	—	3·2		2·4		1·1	
	5×10^{-5}	6·1	+91	6·3	+163	0·58	−47
	1×10^{-4}	7·3	+128	7·7	+220	0·42	−62
2	—	1·0		1·2		3·5	
	5×10^{-5}	1·9	+90	2·2	+83	2·2	−37
3	—	—		—		1·5	—
	5×10^{-5}	—	—	—	—	0·5	−67
	1×10^{-4}	—	—	—	—	0	−100
4	—	0·8		0·6		2·7	
	1×10^{-4}	2·8	+250	2·1	+150	1·3	−52
5	—	1·2		1·1		2·4	
	1×10^{-4}	3·4	+182	2·8	+154	1·4	−42
6	—	2·9		2·4		1·7	
	5×10^{-5}	6·7	+136	5·5	+120	0·9	−47

TABLE IV

EFFECT OF SUBCURATIVE DOSES OF STIBOPHEN ON THE CONCENTRATIONS OF HEXOSEPHOSPHATES IN SCHISTOSOMES

30 mg./kg. of stibophen (0.12% in saline) were administered intraperitoneally to mice infected with *S. mansoni* twice daily at intervals of 8 hr. Infected mice receiving equal volumes of saline served as controls. The worms were removed 1 hr. after the last dose.

Expt.	Total Dose of Stibophen in mg./kg.	F-6-P		G-6-P		HDP	
		Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change
1	Control	2·1		1·8		3·0	
	150	3·2	+52	2·7	+50	1·6	−47
2	Control	1·5		1·2		2·6	
	90	2·3	+53	1·8	+50	1·5	−42
3	Control	1·8		1·5		2·0	
	90	2·7	+50	2·1	+40	1·2	−40
4	Control	0·6		0·6		1·3	
	150	2·2	+256	1·9	+217	0·75	−42
5	Control	2·9		2·6		1·4	
	90	4·1	+41	3·3	+27	0·8	−43
	150	5·7	+97	5·1	+96	0·55	−61
	210	6·1	+110	5·6	+116	0·1	−93

TABLE V

KINETICS OF THE PHOSPHOFRUCTOKINASES OF RABBIT MUSCLE AND OF *SCHISTOSOMA MANSONI*

All concentrations are expressed on the basis of molarity. K_m is the Michaelis–Menten dissociation constant.

	F-6-P		ATP		Mg^{++}		*p*H
	K_m	Optimal Conc.	K_m	Optimal Conc.	K_m	Optimal Conc.	Optimum
Rabbit muscle enzyme ..	$1{\cdot}5\times10^{-4}$	$7{\cdot}5\times10^{-4}$	9×10^{-5}	2×10^{-3}	$1{\cdot}25\times10^{-3}$	5×10^{-3}	8·6
Schistosome enzyme ..	4×10^{-4}	$1{\cdot}5\times10^{-3}$	3×10^{-4}	$3{\cdot}75\times10^{-3}$	9×10^{-4}	$3{\cdot}75\times10^{-3}$	8·3

this consistently occurred following incubation with the antimonial for a period of 1 to 2 hr. In these worms the concentration of HDP was reduced while F-6-P and G-6-P accumulated (Table III). These two esters are in equilibrium through the action of phosphoglucose isomerase, and the changes indicated an inhibition of phosphofructokinase activity during the relatively brief exposure of the parasites to potassium antimonyl tartrate. A similar reduction in the concentration of HDP and an increase in that of F-6-P was observed in worms obtained from mice which had received subcurative doses of stibophen (Table IV). The dosage regime used produced a shift in the distribution of the worms from the mesenteric towards the portal veins; therefore, a slight but demonstrable chemotherapeutic effect had occurred (Schubert, 1948; Standen, 1953).

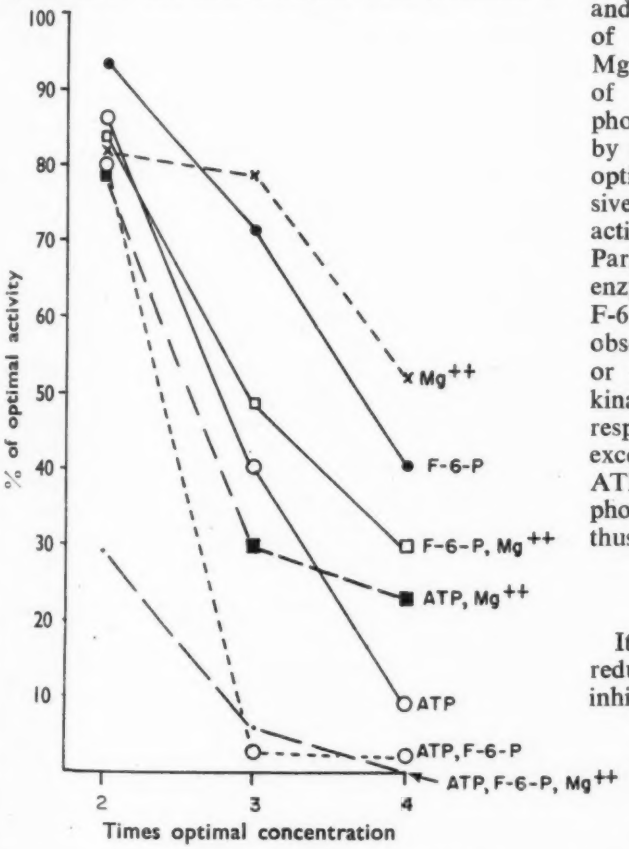

FIG. 3.—Effect of ATP, F-6-P and Mg^{++} in excess of the optimal concentrations on the activity of phosphofructokinase of *S mansoni*.

Because of the critical role of phosphofructokinase in the glycolysis of schistosomes, an attempt was made to determine whether and in what manner this enzyme differs from the one catalysing the same reaction in the host. Measurements of reaction kinetics revealed differences between these two enzymes with regard to their affinities for F-6-P, for ATP and, to a lesser degree, for Mg^{++}, as well as to the effect of *p*H on their activity (Table V). Recently Lardy and Parks (1956) have shown that concentrations of ATP in excess of the molar equivalent of Mg^{++} reduce the activity of phosphofructokinase of rabbit muscle. Similarly, the activity of phosphofructokinase of schistosomes was inhibited by concentrations of ATP which were above the optimal (Fig. 3). On the other hand, while excessive concentrations of Mg^{++} had no effect on the activity of the mammalian enzyme (Lardy and Parks, 1956), they did inhibit the activity of the enzyme of the parasite. The same was true for F-6-P. Inhibition of enzymic activity was observed also when the concentrations of any two or all three constituents of the phosphofructokinase reaction mixture were raised above their respective optima (Fig. 3). It would appear that excessively high intracellular concentrations of ATP, of F-6-P and of Mg^{++} critically reduce the phosphofructokinase activity of schistosomes, thus decreasing the rate of glycolysis of the worms.

DISCUSSION

It was found that trivalent organic antimonials reduce the rate of glycolysis of schistosomes by an inhibition of the activity of phosphofructokinase; this inhibition brings about a decreased formation and thus a lower concentration of HDP which in turn results in a decrease in the activity of aldolase.

Because of the role of phosphofructokinase in determining the rate of glycolysis of *S. mansoni*, it would seem of interest to

point out that the phosphofructokinase of rabbit muscle is much less sensitive than that of schistosomes to the inhibitory effects of antimonials (Mansour and Bueding, 1954). Accordingly, the toxicity of antimonials for the host cannot be ascribed to an inhibition of phosphofructokinase activity. In addition, differences in affinities of the phosphofructokinase of the host and of the parasite for their substrates and in the effect of *p*H on their activities were observed. Finally, in contrast to the rabbit enzyme, excessive concentrations of Mg^{++} markedly inhibited the activity of the worm enzyme. Therefore, it is evident that the enzymes catalysing the same reactions in the parasite and its host are not identical. Because of such differences, opportunities are available to develop compounds which would reduce the activity of the schistosome enzyme without inhibiting the activity of the host enzyme. Such and other differences in the nature of homologous glycolytic enzymes of schistosomes and of mammals have been demonstrated also with hexokinase (Bueding and MacKinnon, 1955a), with lactic dehydrogenase (Mansour and Bueding, 1953 ; Mansour, Bueding and Stavitsky, 1954 ; Henion, Mansour and Bueding, 1955) and with phosphoglucose isomerase (Bueding and MacKinnon, 1955b).

The selective action of trivalent antimonials on phosphofructokinase of *S. mansoni* raises the question about the relationship between this drug-enzyme interaction and the chemotherapeutic effect of antimonials in schistosomiasis. While a multitude of enzymes are affected by drugs, it has been demonstrated only in relatively few instances that such effects are responsible for the pharmacological or chemotherapeutic action of a particular drug. In the opinion of the authors this in no way invalidates the drug-enzyme theory first formulated by Clark (1933), but is ascribed to many experimental difficulties and to frequent neglect in correlating the effects of drugs on isolated biological systems with their action on the intact organism. In a recent review on this subject, Hunter and Lowry (1956) have directed attention to certain requirements which must be met before rigorous proof that a drug acts by inhibiting a particular enzyme can be accepted. In the following, these criteria will be applied to the inhibitory effect of antimonials on phosphofructokinase of schistosomes.

(1) The enzyme concerned should be inhibited in the intact cell. There is evidence for the inhibition of phosphofructokinase by antimonials in the intact worms *in vitro* and *in vivo*: exposure of schistosomes to low concentrations of potassium antimonyl tartrate or administration of subcurative doses of stibophen to the host resulted in an accumulation of F-6-P and in a reduction in the concentration of HDP in the worms ; therefore, under these conditions the substrate of the enzyme was increased and its product was decreased, indicating that the activity of phosphofructokinase within the intact schistosomes was inhibited.

(2) The inhibition of the enzyme should quantitatively explain the effects of the drug. The observations reported in this paper support the conclusion that inhibition of phosphofructokinase is responsible for the reduction in the rate of glycolysis of *S. mansoni*. Previous studies have demonstrated that glycolysis supplies the major, if not exclusive, source of energy for the schistosomes (Bueding, 1950 ; Bueding and Peters, 1951); therefore, it is quite conceivable that inhibition of glycolysis accounts for the death of the worms.

(3) Enzyme inhibition must occur with an amount of drug not greater than that necessary to produce the drug action. Survival of the parasites *in vitro* is reduced from 30 days to approximately 8 hr. by exposure to potassium antimonyl tartrate or to stibophen in concentrations (5×10^{-5} M and 1×10^{-4} M respectively) which produced an inhibition of schistosome phosphofructokinase activity to an extent of 50%. Therefore, there is good agreement between the concentration producing an inhibition of the enzyme and that which exerts a schistosomicidal effect.

(4) Other cell constituents must not bind or inactivate a substantial fraction of the drug. Given concentrations of an antimonial inhibited phosphofructokinase activity of crude schistosome homogenates to the same degree as that of purified preparations of this enzyme ; therefore it appears that there is no significant binding of antimonials by other constituents of schistosome cells.

On the basis of these considerations, it is concluded that inhibition of phosphofructokinase activity can account for the schistosomicidal effect of trivalent organic antimonials. While the possibility cannot be excluded that these drugs may interfere also with other, as yet unknown, mechanisms essential for the survival of the parasite, the present study has revealed a vulnerable point in the metabolism of schistosomes which is susceptible to inhibition by a group of chemotherapeutic agents.

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References

Bueding, E. (1950). *J. gen. Physiol.*, **33**, 475.
— and MacKinnon, J. A. (1955a). *J. biol. Chem.*, **215**, 495.
— — (1955b). Ibid., **215**, 507.
— and Peters, L. (1951). *J. Pharmacol.*, **101**, 210.
Clark, A. J. (1933). *Mode of Action of Drugs on Cells.* Baltimore: Williams and Wilkins.
Cori, G. T., Slein, M. W., and Cori, C. F. (1948). *J. biol. Chem.*, **173**, 605.
Henion, W. F., Mansour. T. E., and Bueding, E. (1955). *Exp. Parasit.*, **4**, 40.
Hunter, F. E., and Lowry, O. H. (1956). *Pharmacol. Rev.*, **8**, 89.
Lardy, H. A., and Parks, R. E., Jr. (1956). *Enzymes: Units of Biological Structure and Function*, p. 584. Ed. O. E. Gaebler. New York: Academic Press.
Ling, K.-H., Byrne, W. L., and Lardy, H. (1955). *Methods in Enzymology*, **1**, p. 306. New York: Academic Press.
Mansour, T. E., and Bueding, E. (1953). *Brit. J. Pharmacol.*, **8**, 431.
— — (1954). Ibid., **9**, 459.
— — and Stavitsky, A. B. (1954). Ibid., **9**, 182.
Schubert, M. (1948). *Amer. J. trop. Med.*, **28**, 137.
Standen, O. D. (1953). *Ann. trop. Med. Parasit.*, **47**, 26.
Taylor, J. F., Green, A. A., and Cori, G. T. (1948). *J. biol. Chem.*, **173**, 591.

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HYPOTHALAMO-NEUROHYPOPHYSIAL INVOLVEMENT IN THE CORTICOTROPHIC ACTION OF ACETYLCHOLINE

BY

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Acetylcholine produced a fall of blood eosinophils and adrenal ascorbic acid in normal rats; these effects did not occur in hypophysectomized animals or in hypophysectomized animals bearing a pituitary homotransplant in the anterior chamber of the eye. Acetylcholine had a striking and long-lasting antidiuretic effect in normal rats, which was abolished by hypophysectomy. In normal dogs, intravenous injections of acetylcholine raised the concentration of posterior pituitary antidiuretic hormone in the cerebrospinal fluid. These results are discussed in connexion with the hypothesis that posterior pituitary antidiuretic hormone may be the neurohumoral substance involved in the hypothalamic control of the anterior pituitary.

Although the secretion of adrenocorticotrophic hormone (ACTH) may be controlled by the nervous system, there is little information available about the details of the mechanism involved.

There is no histological evidence for the direct hypothalamic innervation of the anterior lobe of the pituitary gland. For example, the most recent studies have failed to reveal any hypothalamic nerve fibres in the " pars distalis " (Harris, 1955). The view that the pituitary stalk links the hypothalamus to the adenohypophysis by means of the blood flowing down through the hypophysial portal vessels is supported by many observations. It has been suggested that a humoral substance, liberated by the hypothalamic nerve fibres into the primary plexus of the portal vessels in the median eminence, may reach the anterior lobe and excite the glandular cells (Harris, 1955).

Several suggestions have been put forward as to the nature of this chemical transmitting agent, such as adrenergic substances (Markee, Sawyer, and Hollinshead, 1948), histamine (Harris, Jacobsohn, and Kahlson, 1952), serotonin (Bertelli, Cantone, and Martini, 1954), substance P (Pernow, 1953), lipoid or lipoprotein present in hypothalamic extracts (Slusher and Roberts, 1954), and neurosecretory material produced in the hypothalamo-posthypophysial system (Benoit and Assenmacher, 1953; Palay, 1953; Rothballer, 1953).

In previous work, evidence was obtained supporting the view that the antidiuretic hormone (ADH) produced in hypothalamic nuclei may be considered as the neurohumoral substance involved in the hypothalamic control of the anterior pituitary (Martini and Morpurgo, 1955; Martini and De Poli, 1956; Martini, De Poli, and Curri, 1956).

The present investigation is concerned with the possible rôle played by acetylcholine (ACh) in the ACTH stimulating mechanism. Many observations seem to suggest that ACh may be an important factor in the pituitary activation by neurotropic stimuli. ACh occurs in high concentration in the hypothalamus (Macintosh, 1941; Feldberg, Harris, and Lin, 1951). The synthesis of ACh occurs in hypothalamic nuclei (Feldberg and Vogt, 1948). Moreover, many emotional stimuli associated with ACTH liberation may raise the ACh concentration in blood (Diethelm, Fleetwood, and Milhorat, 1950).

Experiments were planned to ascertain whether ACh could stimulate the release of ACTH from the anterior pituitary and to find whether the adrenocorticotrophic effect of ACh could be considered to be due to a direct action on hypophysial glandular cells rather than an indirect action involving the stimulation of the hypothalamic-neurohypophysial system.

METHODS

Animals and Drugs.—Male rats of the Sprague-Dawley strain, weighing 150 to 200 g., were used in the ACTH-releasing experiments and in the anti-

diuretic assays; they were maintained on commercial diet and tap water. Dogs were used in the experiments studying the action of ACh on the concentration of ADH in the cerebrospinal fluid.

ACh was usually dissolved in 0.9% NaCl solution; control animals were injected with corresponding volumes of 0.9% NaCl solution.

The significance of the differences between the means of the results obtained was calculated by Student's "*t*" test.

Hypophysectomy and Pituitary Transplantation.—Hypophysectomy was performed in rats by the standard parapharyngeal approach; the hypophysectomized animals were used 5 days later.

In a group of animals the procedure of introducing adenohypophysial transplants into the anterior chamber of the right eye was carried out under light ether anaesthesia, animals of the same sex and weight serving as donors; in this group, hypophysectomy was performed two days after grafting. Tests on the grafted rats were begun 50 days after hypophysectomy, ACh being given either by intraperitoneal injection or by local application to the graft-containing eyes by means of subconjunctival injections.

Eosinophils and Ascorbic Acid Depletion Test.—Tests were performed in normal rats, in hypophysectomized rats and in hypophysectomized rats bearing adenohypophysial homotransplants in the anterior chamber of the eye.

Immediately before, 2, and 4 hr. after giving intraperitoneal injections of ACh, direct counts of the eosinophils in the blood were made by the method of Recant, Forsham and Thorn (1948) on blood samples taken from the tail. Although it is recognized that this test is not a specific index of increased adrenocortical function, it is certainly very sensitive and was therefore adopted as a guide.

The results were subsequently confirmed with the ascorbic acid depletion test (Sayers, Sayers and Woodbury, 1948). The rats were killed by decapitation 3 hr. after treatment, the adrenals removed, freed from excess adipose and connective tissue, weighed on a torsion balance, and their content of ascorbic acid was estimated by the method of Roe and Kuether (1943).

Antidiuretic Test.—The antidiuretic action of ACh was studied both in normal and in hypophysectomized rats, after hydration according to Ginsburg's (1951) procedure. The percentage water excretion 60, 90 and 120 min. after the intraperitoneal injection of ACh was calculated; this is inversely related to the antidiuretic power of the drug injected.

Antidiuretic Hormone Level in Dog Cerebrospinal Fluid.—The antidiuretic power of dog cerebrospinal fluid was determined on samples obtained from unanaesthetized animals by suboccipital puncture. The first sample (1 ml.) was obtained just before the intravenous injection of ACh in the test animals, and of 0.9% NaCl solution in the control animals. The second sample (1 ml.) was obtained 5 min. later through the same needle which had remained inserted. The antidiuretic power of these samples was tested by injecting subcutaneously 0.1 ml. of the cerebrospinal fluid into hydrated rats according to Ginsburg's (1951) procedure. Inactivation with sodium thioglycollate (Van Dyke, Chow, Greep and Rothen, 1942) was carried out in order to make sure that the antidiuretic activity of cerebrospinal fluid samples was due to posterior pituitary ADH.

Results

Effect of Acetylcholine on the Number of Circulating Eosinophils and on Adrenal Ascorbic Acid in Normal, Hypophysectomized, and Hypophysectomized Rats Bearing a Pituitary Graft in the Anterior Chamber of the Eye.—In normal rats, the intraperitoneal injection of ACh (1 mg./rat) was followed by a highly significant fall ($P<0.001$) in the number of the circulating eosinophils; no corresponding change in the eosinophil blood count occurred in hypophysectomized or in grafted animals (Fig. 1).

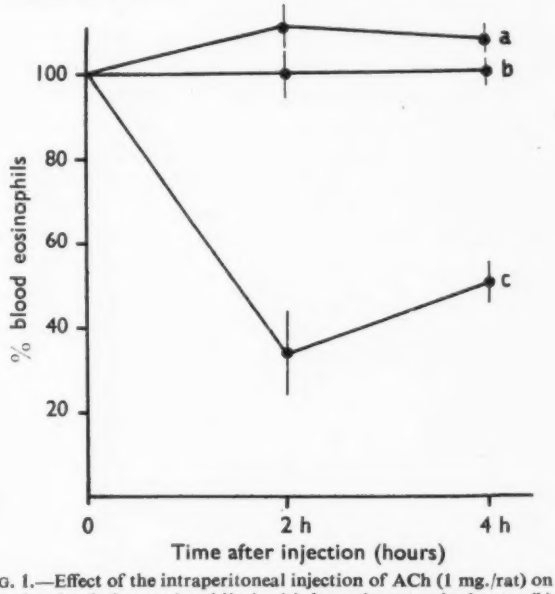

FIG. 1.—Effect of the intraperitoneal injection of ACh (1 mg./rat) on the circulating eosinophils in (a) hypophysectomized rats, (b) hypophysectomized rats bearing intraocular pituitary grafts, and (c) normal rats. Each point represents the mean of 10 experiments; the vertical lines indicate the standard error.

Similar results were obtained with the ascorbic acid depletion test. In normal rats, the intraperitoneal injection of ACh (1 mg./rat) resulted in a significant fall ($P<0.001$) in adrenal ascorbic acid. In hypophysectomized rats the same dose of ACh did not cause any depletion of ascorbic acid. Both by the intraperitoneal route (1 mg./rat), and by subconjunctival injection into the graft-containing eyes (30 μg./rat), ACh was ineffective in the grafted animals (Table I).

TABLE I

EFFECT OF INTRAPERITONEAL AND SUBCONJUNCTIVAL INJECTIONS OF ACETYLCHOLINE ON ADRENAL ASCORBIC ACID IN NORMAL RATS, HYPOPHYSECTOMIZED RATS AND HYPOPHYSECTOMIZED RATS BEARING INTRAOCULAR PITUITARY GRAFTS

No. of Animals	Treatment	Adrenal Ascorbic Acid (mg./100 g. Tissue ± Standard Error)		
		Normal	Hypophysectomized	Hypophysectomized and Grafted
10	NaCl 0·9% ..	432·1±25·1	422·5±18·5	452·3±15·0
10	Acetylcholine (1 mg./rat intraperitoneally)	325·0±13·5	445·0±22·4	427·5± 8·0
10	Acetylcholine (30 μg./rat subconjunctivally)	—	—	458·0±11·5

These experiments indicated that ACh exerted a marked corticotrophic effect, which was mediated through the activation of the anterior pituitary gland. The results obtained in the hypophysectomized animals bearing pituitary transplants showed that adenohypophysial cells did not respond in a direct way to ACh administration. Since the responsiveness of the pituitary gland to the drug depended upon the integrity of normal hypothalamo-hypophysial pathways, the action of ACh must take place at a higher level, probably in the hypothalamus. The stimulation of the hypothalamic-neurohypophysial system by ACh was then studied.

Effect of Acetylcholine on Water Diuresis in Normal and Hypophysectomized Rats.—ACh, when injected intraperitoneally (1 mg./rat) into hydrated normal rats, has a striking and long-lasting antidiuretic effect (Fig. 2). The drug has no antidiuretic effect in hypophysectomized rats in which, however, the elimination of the water-load is slower than in normal animals. As these two results appeared very similar in their characteristics to those obtained after giving posterior pituitary gland preparations, it seemed that the antidiuretic effect observed after ACh administration might have been due to a discharge of antidiuretic hormone from the hypothalamic-neurohypophysial system. This hypothesis was tested in the following experiments in which the concentration of ADH in the cerebrospinal fluid was measured in normal dogs before and 5 min. after giving intravenous injections of ACh.

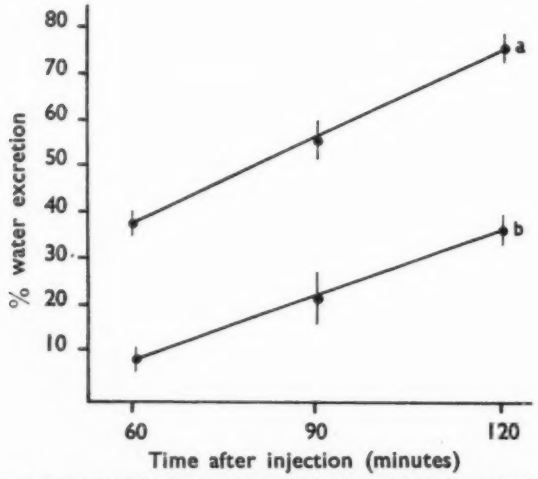

FIG. 2.—Effect of the intraperitoneal injection of ACh (1 mg./rat) on the water excretion of hydrated normal rats. (a) Rats injected with 0.9% NaCl solution. (b) Rats injected with ACh. Each point represents the mean of 10 experiments; the vertical lines indicate the standard error.

Effect of Acetylcholine on the Cerebrospinal Fluid Levels of ADH in Normal Dogs.—Fig. 3 summarizes the results obtained on injecting subcutaneously three groups of hydrated rats (*a*) with 0.1 ml. of 0.9% NaCl solution, (*b*) with 0.1 ml. of cerebrospinal fluid taken from three normal dogs before ACh administration, and (*c*) with 0.1 ml. of cerebrospinal fluid taken from the same three dogs 5 min. after the intravenous injection of 2 mg./kg. of ACh. The antidiuretic power of the cerebrospinal fluid was significantly enhanced after ACh administration.

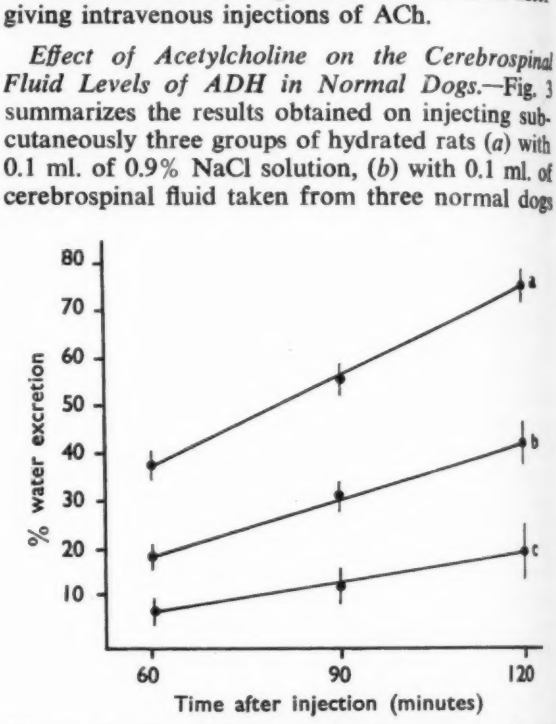

FIG. 3.—The water excretion in hydrated normal rats after the subcutaneous injection of cerebrospinal fluid (0.1 ml./rat) taken from normal dogs before and 5 min. after the intravenous injection of 2 mg./kg. of ACh. (a) Rats injected with 0.9% NaCl solution. (b) Rats injected with cerebrospinal fluid taken before ACh administration. (c) Rats injected with cerebrospinal fluid taken after ACh administration. Each point represents the mean of 15 experiments performed with cerebrospinal fluid taken from 3 dogs; the vertical lines indicate the standard error.

In our experiments the concentration of ADH in the cerebrospinal fluid of normal dogs (about

7.5 mU./ml.) was much higher than that found by Vogt (1953), namely <2.5 mU./ml.

After incubation with sodium thioglycollate, the antidiuretic activity of cerebrospinal fluid, withdrawn before or after giving ACh, was abolished in the same way as that of the posterior pituitary hormone after similar treatment.

In two dogs treated with 0.9% NaCl solution instead of with ACh, no enhancement of the cerebrospinal fluid level of ADH was observed.

Discussion

The results reported here indicate that ACh can stimulate the anterior pituitary to discharge ACTH. This is in agreement with the observation of Hofmann-Credner (1953) that ACh injections produced eosinopenia in human beings. Similarly, Guillemin (1955) found that methacholine lowered the adrenal ascorbic acid in normal rats. Working on the same lines, Dordoni and Fortier (1950) and Dordoni and Timiras (1952) have shown that in intact animals a single injection of eserine depleted the adrenal ascorbic acid and that prolonged treatment with eserine produced marked hypertrophy of the adrenals and atrophy of the thymus.

These studies of the ability of parasympathomimetic drugs to release ACTH have an interesting parallel in those of Taubenhaus and Soskin (1941), which showed that injection of ACh resulted in the production of pseudopregnancy in the rat, that is, in the release of luteotrophic substance (lactogenic hormone) from the anterior pituitary. According to Markee, Everett, and Sawyer (1952) ACh also plays an important rôle in the liberation of enough luteinizing hormone (L.H.) to cause ovulation.

As to the site and mode of action of ACh as an ACTH-releaser, the present experiments have clearly shown that it has no direct stimulating action on adenohypophysial glandular cells; ACh is thus unlikely to be the substance released by hypothalamic neurones into the hypophysial portal vessels under the influence of stress, and it is not responsible for an increase in the pituitary secretion of ACTH above the resting state. In agreement with these results, Feldberg and Vogt (1948) have shown that there are only small amounts of the ACh-forming enzymes in the pituitary gland; and they concluded that no cholinergic fibres originate from the cells in the supra-optic region.

As the integrity of normal hypothalamo-hypophysial connexions was needed for the action of ACh, it seemed that the drug might act on these pathways at the hypothalamic level. We have shown that, in rats and dogs after the administration of ACh, there is a stimulation of hypothalamic centres of neurones, which leads to the release of antidiuretic hormone from the hypothalamo-neurohypophysial system.

Using adrenaline, which also can release ACTH, Martini and Rovati (1952a, 1952b, 1956) obtained similar results.

Evidence that ACh may act on hypothalamic nuclei or neurones has been obtained also by Pickford and Watt (1951) and by Abrahams and Pickford (1954). These authors have shown that intravenous and intracarotid injections of ACh produced a marked antidiuretic effect in normal hydrated dogs, but failed to inhibit water diuresis after the production of diabetes insipidus by suitably placed hypothalamic lesions. ACh, injected directly into the supra-optic nuclei of normal dogs during a water diuresis, resulted in a marked inhibition of the rate of urine flow (Pickford, 1947); such an inhibition may be prolonged by adding eserine to the solution injected (Pickford, 1947). Eserine and DFP, when injected alone, produce a similar long-lasting antidiuretic effect (Duke, Pickford, and Watt, 1950); eserine and DFP when injected into the supra-optic nuclei of dogs anaesthetized with chloralose led to a release of oxytocin and to an increase in size of spontaneous uterine contractions (Abrahams and Pickford, 1956).

These experiments support the hypothesis that the action of ACh on the anterior pituitary is mediated through the release of antidiuretic hormone, or some related compound liberated at the same time as antidiuretic hormone, into the hypophysial portal vessels.

The early experiments of Martini and Morpurgo (1955), of Martini and De Poli (1956), and of Martini, De Poli, and Curri (1956), as well as more recent investigations, indicated that posterior pituitary preparations do, indeed, stimulate the release of ACTH. Sayers (1956) has recently shown that a commercial antidiuretic hormone preparation (Pitressin, Parke, Davis) and a more purified vasopressin sample (Du Vigneaud's AVN-5) exhibited a marked action causing the discharge of ACTH. McDonald and Weise (1956) and Shibusawa, Saito, Fukada, Kawai, and Yoshimura (1955) have shown that, in man, plasma 17-hydroxycorticosteroid levels were elevated during infusions of both commercial pitressin and purified antidiuretic hormone preparations (Du Vigneaud's arginine- and lysine-vasopressin). Working on the same lines, McCann and Brobeck (1954) have reported that lesions of the supra-optico-hypo-

physial tract, which produced diabetes insipidus, blocked the release of ACTH in response to stressor agents. In rats which, as a result of lesions in the median eminence, no longer responded to other stressors, ACTH secretion can be elicited by the administration of pitressin.

The physiological significance of the power of ACh to release ACTH is not yet clear; it should be remembered in this connexion that Guillemin (1955) has shown that in rats treated with atropine the release of ACTH which normally follows the injection of parasympathomimetic drugs was completely prevented, while ACTH was still liberated by systemic stress and neurotropic stimulation.

References

Abrahams, V. C., and Pickford, M. (1954). *J. Physiol.*, **126**, 329.

—— —— (1956). Ibid., **133**, 330.

Benoit, J., and Assenmacher, I. (1953). *Arch. Anat. micr.*, **42**, 334.

Bertelli, A., Cantone, G., and Martini, L. (1954). *Atti Soc. lombarda Sci. med. biol.*, **9**, 10.

Diethelm, O., Fleetwood, M. F., and Milhorat, A. T. (1950). *Proc. Ass. Res. nerv. Dis.*, **29**, 262.

Dordoni, F., and Fortier, C. (1950). *Proc. Soc. exp. Biol. N.Y.*, **75**, 815.

—— and Timiras, P. S. (1952). *J. Pharmacol.*, **106**, 381.

Duke, H. N., Pickford, M., and Watt, J. A. (1950). *J. Physiol.*, **111**, 81.

Feldberg, W., Harris, G. W., and Lin, R. C. Y. (1951). Ibid., **112**, 400.

—— and Vogt, M. (1948). Ibid., **107**, 372.

Ginsburg, M. (1951). *Brit. J. Pharmacol.*, **6**, 411.

Guillemin, R. (1955). *Endocrinology*, **56**, 248.

Harris, G. W. (1955). *Neural Control of the Pituitary Gland*, 1st ed., p. 7. London: Edward Arnold.

—— Jacobsohn, D., and Kahlson, G. (1952). *Ciba Foundation Colloquia on Endocrinology*, **4**, 186.

Hofmann-Credner, D. (1953). *Klin. Med.*, **8**, 215.

McCann, S. M., and Brobeck, J. R. (1954). *Proc. Soc. exp. Biol. N.Y.*, **87**, 318.

McDonald, R. K., and Weise, V. K. (1956). Ibid., **92**, 481.

Macintosh, F. C. (1941). *J. Physiol.*, **99**, 436.

Markee, J. E., Everett, J. W., and Sawyer, C. H. (1952). *Recent Progr. Hormone Res.*, **8**, 139.

—— Sawyer, C. H., and Hollinshead, W. H. (1948). Ibid., **2**, 117.

Martini, L., and De Poli, A. (1956). *J. Endocrin.*, **13**, 229.

—— —— and Curri, S. (1956). *Proc. Soc. exp. Biol. N.Y.*, **91**, 490.

—— and Morpurgo, C. (1955). *Nature, Lond.*, **175**, 1127.

—— and Rovati, V. (1952a). *Atti Soc. lombarda Sci. med. biol.*, **7**, 393.

—— —— (1952b). *Boll. Soc. ital. Biol. sper.*, **28**, 1593.

—— —— (1956). *Arch. int. Pharmacodyn.*, **104**, 365.

Palay, S. L. (1953). *Amer. J. Anat.*, **93**, 107.

Pernow, B. (1953). *Nature, Lond.*, **171**, 746.

Pickford, M. (1947). *J. Physiol.*, **106**, 264.

—— and Watt, J. A. (1951). Ibid., **114**, 333.

Recant, L., Forsham, P. H., and Thorn, G. W. (1948). *J. clin. Endocrin.*, **8**, 589.

Roe, J. H., and Kuether, C. A. (1943). *J. biol. Chem.*, **147**, 399.

Rothballer, A. B. (1953). *Anat. Rec.*, **115**, 21.

Sayers, G. (1956). *Fed. Proc.*, **15**, 162.

Sayers, M. A., Sayers, G., and Woodbury, L. A. (1948). *Endocrinology*, **42**, 379.

Shibusawa, K., Saito, S., Fukada, M., Kawai, T., and Yoshimura, F. (1955). *Endocr. jap.*, **2**, 47.

Slusher, M. A., and Roberts, S. (1954). *Endocrinology*, **55**, 245.

Taubenhaus, M., and Soskin, S. (1941). Ibid., **29**, 958.

van Dyke, H. B., Chow, B. F., Greep, R. O., and Rothen, A. (1942). *J. Pharmacol.*, **74**, 190.

Vogt, M. (1953). *Brit. J. Pharmacol.*, **8**, 193.

Brit. J. Pharmacol. (1957), **12**, 171.

ANTIMALARIAL ACTIVITY OF HYDROXY-SUBSTITUTED NAPHTHALENE COMPOUNDS

BY

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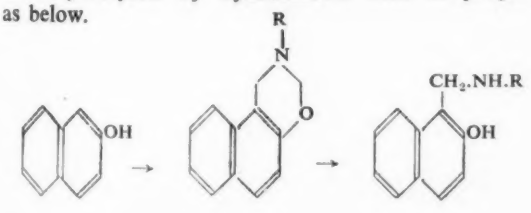
A new series of mono- and di-hydroxy substituted naphthalene compounds was synthesized and found to possess antimalarial activity against *P. gallinaceum* infection of young chicks. A representative compound with a high degree of activity was chosen for extensive testing against other malarial species and for pharmacological investigation. The formula of this compound, number 377C54, was 1:6-dihydroxy-2:5-bis(*cyclo*hexylaminomethyl)naphthalene dihydrochloride. Effective doses against *P. gallinaceum* in chicks, *P. berghei* in mice and *P. cathemerium* in canaries were 2.3, 4.0, and about 6 mg./kg. respectively. Compound 377C54 acted rapidly against the parasitaemia of *P. gallinaceum* in chicks and *P. knowlesi* in a rhesus monkey. Parasiticidal activity remained in the blood of chicks for a long time after a single oral dose. The drug can be estimated by the production of colour on coupling with diazotized *p*-nitroaniline. Drug concentrations in blood from chicks and humans rose rapidly after oral administration. In tissues from chicks, particularly liver and lung, the drug persisted for a long period. An unsuccessful attempt was made to induce resistance to 377C54 in a strain of *P. gallinaceum*. Extensive pharmacological investigation showed that 377C54 possessed no special pharmacological properties.

Several derivatives of 1- and 2-naphthol were tested for antimalarial activity in the American wartime screening programme (Wiselogle, 1946). One member of the series, which showed activity against laboratory malaria infections, SN 6520 [2-(dimethylaminomethyl)-1-naphthol], was given clinical trial against blood-induced *Plasmodium vivax* infections. It was found to be inactive at doses of 2 g. daily. No tests were made for either prophylactic or curative activity.

The experiments recorded here are the result of the observation that 1-*cyclo*hexylaminomethyl-2-naphthol (371C52) showed antimalarial activity against blood-induced *P. gallinaceum* infection in young chicks.

METHODS

The compounds were made by the method described by Burke, Kolbezen, and Stephens (1952). The corresponding naphthols were reacted with formaldehyde and a primary amine to give an oxazine which was then hydrolysed by hydrochloric acid in propanol, as below.



Screening and assaying of new compounds were carried out using *P. gallinaceum* infections of 5- or 12-day-old chicks. The methods used were those previously described by Falco, Goodwin, Hitchings, Rollo, and Russell (1951). Where compounds were also tested against *P. berghei* in mice the technique of the same authors was used. Where only a screening test was carried out the compound was awarded a "score" according to the following key:

0=No activity at 100 mg./kg.
1=Slight activity at 100 mg./kg.
2=Active at doses between 100 and 10 mg./kg.

A dose was considered to be "active" when the mean % parasitaemia of the treated group of animals was lowered to 1% or less of the mean % parasitaemia of untreated controls. When an assay was carried out, the median effective dose (ED50) was determined. This is defined as the dose which reduces parasitaemia to 50% of the mean parasitaemia of untreated controls. A 3- or 4-dose assay was used as described by Rollo (1952).

Compound 377C54 was also tested against blood-induced infections of *P. cathemerium* in canaries and *P. knowlesi* (nuri strain) in a rhesus monkey. Canaries were inoculated intravenously with approximately 25×10^6 parasitized red blood cells, and were given a course of treatment lasting four days as in the tests using *P. gallinaceum*. The monkey was infected by the intraperitoneal injection of 3 ml. of infected citrated blood and was given single oral doses to assess

the effect of the drug on a moderately high parasitaemia. The drug was given orally in solution. The blood was examined by prolonged search of thin films. Thick films were not made. Single oral doses were also given to chicks infected with *P. gallinaceum* to determine the speed of action of the compound on an established infection. The appearance of the parasites at various times after dosing was assessed from an examination of stained blood films.

In some experiments single doses of drug were given to uninfected chicks and the persistence of the substance in the blood was assessed by challenge at intervals by intravenous inoculation of parasitized red blood cells. The details of this test are as follows: (i) The dose of compound 377C54 used was 200, 50, or 12.5 mg./kg. (ii) Young chicks were infected by an inoculum of *P. gallinaceum* containing 5×10^7, 5×10^6, or 5×10^5 parasitized red cells. (iii) The time interval between dosing and inoculation was 24, 48, or 96 hr. The chicks were examined daily for 11 days after inoculation and the mean number of days free from parasites for each group was estimated using the formula,

$$(1/N_0)(\tfrac{1}{2}N_0 + N_1 + N_2 + \ldots\ldots N_{n-1} + \tfrac{1}{2}N_n)$$

where N_n = number of chicks free from parasites on day n and n = length of experiment in days.

In an attempt to induce resistance to the compound, a substrain from the normal *P. gallinaceum* strain was treated with subcurative amounts over a period of 33 weeks. The strain was passaged many times during the period. Normal assays were carried out during and at the end of the time, to compare the responses of the treated strain with those of the untreated parent strain. Estimations were made of the concentration in the blood and tissues of chicks and in the blood of human volunteers after a single oral dose of a selected member of the series. Before extraction with redistilled ether, the blood was laked in ten times its own volume with 1% alcohol in distilled water; tissues were homogenized. The extraction and all subsequent operations were conducted by the light of a red photographic safelight. The drug was extracted into ether after the *p*H of the sample had been adjusted to 8 with phosphate buffer. The drug was then taken into 5 ml. N/10-HCl and a colour developed with diazotized *p*-nitroaniline. The intensity of the colour was immediately estimated by reading at 465 mμ in a Unicam Spectrophotometer (Model SP 350).

Results

Multiple Dose Experiments

Activity of Some 1-Hydroxynaphthalene Derivatives.—In the examples tested, compounds with a substituted amino methyl group in the 2-position had moderately high antimalarial activity, such as the *cyclo*hexylamino- (ED50 40 mg./kg.) and *n*-butylamino- (score 2) compounds. However, if the aliphatic chain was considerably lengthened, as in octylamino-, activity was lost.

Methoxy-substitution in position 5 had little effect on the activity of the 2-*cyclo*hexylamino-compound, but in position 4 reduced activity. A chlorine atom in the 4-position did not affect activity.

Substances with heterocyclic basic groups in position 2, such as the pyrrolidinomethyl- and piperidinomethyl- compounds, also showed activity (score 2). The non-basic pyrrolidide, which is insoluble, was inactive.

Activity of 2-Hydroxynaphthalene Derivatives.—Compounds with a substituted aminomethyl group in position 1 were active provided the substituent was sufficiently large. The unsubstituted and the methyl- and *iso*propyl- substituted compounds were inactive. Of the compounds tested, optimum activity was found in the aliphatic series with *iso*pentyl (score 2). *cyclo*Pentyl, *cyclo*hexyl, and *cyclo*heptyl derivatives had about the same activity (ED50 70, 98, and 83 mg./kg. respectively). It is interesting that the only tertiary amino compound tested, 1-di-*n*-propylaminomethyl-2-hydroxynaphthalene, was inactive.

Substitution of the hydroxy group in the 1-*cyclo*hexylaminomethyl compound by methoxy resulted in a considerable increase in activity (ED50 58 mg./kg.). Increase of activity was also observed when the unsubstituted ring of the naphthalene nucleus was reduced (score 2). Substitution with methyl, methoxy, or hydroxymethyl groups in position 3 of the naphthalene nucleus led to compounds of lower activity.

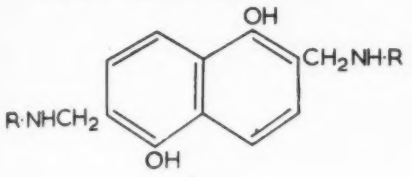
In the 1-*cyclo*hexylaminomethyl series several compounds were prepared with substituents in the *cyclo*hexyl ring. The effect of a methyl group depended on its position: 2-methyl (score 1) gave lower activity than either 3-methyl or 4-methyl (both score 2). Chlorine in position 2 reduced, whereas hydroxyl increased, activity.

Derivatives of three dihydroxynaphthalenes have been investigated (Table I). Owing to instability only one example (382C54) of the 1:5-dihydroxy compounds was prepared. This was less active than the 1:6 and 2:6 isomerides.

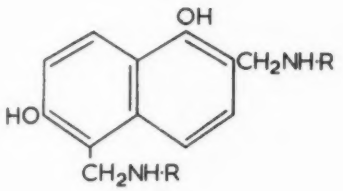
Among the members of the 1:6-dihydroxy series were found the most active of all the compounds tested. Activity depended on the length of the alkyl substituent of the amino group, the *n*-propyl and the *n*-butyl substituted compounds being the most active of those tested. Lengthening of the chain to 8 carbon atoms (356C55) resulted in a marked decrease in activity. *cyclo*Pentyl, *cyclo*hexyl-, and *cyclo*heptyl- substituted compounds were highly active.

TABLE I

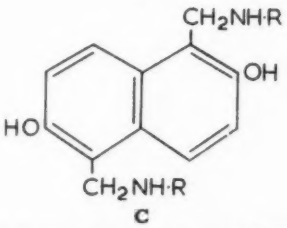
THE ANTIMALARIAL ACTIVITY OF DIHYDROXY-BIS(R. AMINOMETHYL)NAPHTHALENES



A



B



C

The asterisk denotes the mean of several determinations.

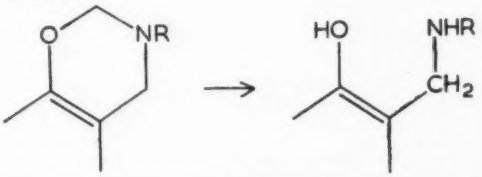
Reference No.	R	Score of Activity or ED50's in Parentheses (mg./kg.)
	Group A 1 : 5-*di*(OH)-2 : 6-*bis*(R.NH.CH_3)	
382C54	*cyclo*Hexyl	(8·1)
	Group B 1 : 6-*di*(OH)-2 : 5-*bis*(R.NH.CH_3)	
672C55	Ethyl	(6·1)
608C55	*n*-Propyl	(1·4)
325C55	*n*-Butyl	(1·5)
356C55	*n*-Octyl	(35·5)
322C55	*cyclo*Pentyl	(1·8)
377C54	*cyclo*Hexyl	(2·3)*
372C55	*cyclo*Heptyl	(2·7)
	Group C 2 : 6-*di*(OH)-1 : 5-*bis*(R.NH.CH_3)	
66C56	Methyl	2
115C56	Ethyl	(10)
97C56	*n*-Propyl	(4·6)
91C56	*iso*-Propyl	(3·9)
26C56	*n*-Butyl	(3·2)
67C56	*n*-Pentyl	(3·4)
322C56	*n*-Hexyl	(8·9)
323C56	*n*-Heptyl	(89)
216C54	*cyclo*Hexyl	(4·3)

Members of the 2 : 6-dihydroxy series were less active than the corresponding compounds in the 1 : 6-dihydroxy series. The *n*-butyl compound was the most active.

Since all the aminomethylnaphthols were prepared from the corresponding oxazines, it was thought of interest to examine the activity of some of the oxazines themselves. Assays were carried out with two of the oxazines and the corresponding naphthalenes and the following results obtained:

	ED50 (mg./kg.)
1-hydroxy-2-*cyclo*hexylaminomethylnaphthalene	40
3-*cyclo*hexyl-3 : 4-dihydro-2H-naphth(2 : 1,e)-*m*-oxazine	51
1 : 5-di(hydroxy)-2 : 6-bis(*cyclo*hexylaminomethyl) naphthalene	8.1
2 : 8-*cyclo*hexyl-1 : 2 : 3 : 4 : 7 : 8 : 9 : 10-octahydro-2 : 8-diaza-4 : 10-dioxachrysene	7.8

It is obvious that the ED50's of the related pairs were of the same order—indeed those of the latter pair, allowing for experimental error, can be said to be the same. The evidence, therefore, points to a similarity of action in the paired compounds, and it seems probable that the oxazines break down *in vivo* to the corresponding active compound thus:



Because of its marked antimalarial action, compound 377C54 [1 : 6-dihydroxy-2 : 5-bis(*cyclo*hexylaminomethyl)naphthalene dihydrochloride] was selected to undergo extensive testing against laboratory malarial infections and pharmacological investigation.

The results in Table II show that the high activity shown against *P. gallinaceum* in chicks is also shown against *P. berghei* in mice and *P. cathemerium* in canaries.

TABLE II

ASSAY OF COMPOUND 377C54 AGAINST THREE SPECIES OF MALARIAL PARASITE

Species	Drug	Dose (mg./kg.)	Mean % Parasitaemia	ED50 (mg./kg.)
P. gallinaceum ..	377C54	5 3·54 2·5	<1 21 76	3·3
	Untreated controls	—	79	—
P. berghei ..	377C54	6 3 1·5	<1 29 55	4·0
	Untreated controls	—	33	—
P. cathemerium	377C54	10 2·5	0 19	*ca.* 6
	Untreated controls	—	18	—

Single Dose Experiments

Effect of a Single Oral Dose on High Parasitaemia

P. gallinaceum *in Young Chicks.*—Single oral doses of 10 mg. base/kg. of chloroquine and of 377C54 affected the parasites of an established infection of *P. gallinaceum* in chicks after 4 hr., and after 8 hr. nearly all the parasites in stained smears showed degenerative changes. These changes appeared in trophozoites; young merozoites and mature schizonts seemed to be unaffected. Doses of 2.5 mg./kg. of either compound had no effect upon the morphology of the parasites. The new compound had a speed of action equal to that of chloroquine.

P. knowlesi *in a Rhesus Monkey.*—Three days after inoculation, examination of the monkey's blood showed the presence of numerous parasites. The first dose was then administered; subsequent doses were given whenever parasites again appeared in the blood in any number.

The rapidity of action of the drug which became evident from the experiments with *P. gallinaceum* was also noted with *P. knowlesi.* After 50 mg./kg. all parasites had disappeared from the blood within 17 hr. and remained absent for 10 days. With 20 and 10 mg./kg., parasites disappeared within 24 hr. and remained absent for 7 and 3 to 5 days respectively. When 5 mg./kg. was given, a single parasite was seen 24 hr. later after prolonged search. The blood was free from parasites for 2 days.

It seems unlikely that immunity to the infection influenced the later results, because the length of time free from parasitaemia was roughly proportional to the dose used, and because when immunity was established the animal was free from parasites for many weeks.

This strain is known to be extremely virulent in rhesus monkeys: if left untreated, the parasites rapidly multiply and the monkey dies in 7 to 14 days.

Persistence of Parasiticidal Activity in Blood after a Single Oral Dose

Parasiticidal activity remained in the blood for a considerable length of time after a single oral dose, as is shown in Table III. Two explanations can be put forward. Either the drug, or a possible metabolite, remained in the blood perhaps bound to plasma protein, or, as is well known to be the case with chloroquine, the drug was first taken up by the tissues and then slowly liberated.

In order to obtain more evidence on these points chemical estimations of blood and tissue concentrations were carried out.

TABLE III

MEAN NUMBER OF DAYS FREE FROM INFECTION OF *P. GALLINACEUM* AFTER A SINGLE DOSE OF 377C54 RELATED TO THE INTERVAL BETWEEN DOSE AND INOCULATION

Each figure in the Table represents the mean number of days free from infection, and, where this figure is greater than that for the corresponding untreated control group, it appears in bold type.

Interval Between Dose and Inoculation (hr.)	Dose 377C54 (mg./kg.)	No. of Days Free from Infection		
		Inoculum (Approx. Number Parasitized Red Cells)		
		5×10^7	5×10^5	5×10^3
24	200	**9·5**	**10·8**	**11**
	50	**9**	**9**	**>11**
	12·5	<4	**5·8**	**>11**
	—	<4	<4	**7·3**
48	200	**8·6**	**>10**	**>11**
	50	**7·3**	**9·2**	**>11**
	12·5	<4	4	**8·3**
	—	<4	<5	**8·5**
96	200	**6·5**	**8·8**	**>11**
	50	<4	<5	**8**
	12·5	<4	<5	**8·8**
	—	<4	<5	**>9**

Estimation of 377C54 in Blood and Tissues

Blood and Tissues from Chicks given Single Oral Doses of 377C54

Chicks, given 100 mg./kg. of 377C54, were killed at various times after the dose. Table IV shows a series of results obtained when the drug was extracted from blood and liver.

TABLE IV

CONCENTRATION OF 377C54 AFTER SINGLE ORAL DOSE OF 100 MG./KG. IN THE CHICK

Hr. after Dose	Blood Concentration (mg.%)	Liver Concentration (mg./Liver)
¼	0·10	0·01
½	0·16	0·03
1	0·23	0·12
2	0·27	0·18
4	0·07	0·30
7	0·15	0·41

Estimations of drug from tissues taken from a chick weighing 65 g. which had been dosed with 10 mg./kg. of 377C54 24 hr. previously showed that no drug could be recovered from blood, brain, heart, kidney, muscle, or spleen, but that liver and lung contained totals of 40 and 2.0 μg. respectively.

A further batch of chicks was dosed with 10 mg./kg. These were killed at 3, 7, and 11 days afterwards and the concentration of 377C54 in liver and lungs estimated. At the end of these periods the lungs contained 4, 7, and 5 μg. of the drug; at the end of 3 days the liver contained 5 μg., but none could be recovered after 7 days.

The concentration of drug in the blood reached a peak in about 2 hr. and thereafter slowly decreased. The maximal concentration of drug in the liver was attained more slowly, and 3 days after the comparatively small dose of 10 mg./kg. there was still an appreciable amount of drug present. Measurable quantities of drug were still present in lung 11 days after a single oral dose.

Blood and Urine from Human Volunteers

A dose of 600 mg. of 377C54 was taken by mouth by each volunteer. From the results given in Table V it may be concluded that the drug

TABLE V

CONCENTRATION OF 377C54 IN HUMAN BLOOD AND URINE AFTER SINGLE ORAL DOSE OF 600 MG.

Individual	Blood		Urine	
	Interval (hr.)	Concentration 377C54 (μg.%)	Interval (hr.)	Total 377C54 in Sample (μg.)
J. H. ..	1	37·5	2¼	Nil
	2	72·5	3	28·4
	4	66·6	5½	48·8
			7	41·3
H. R. ..	1	75·0	2¼	Nil
	2	58·0	6	41·5
	4	30·0	8½	5·4
	8	Nil		

rapidly attained a peak of concentration in the blood, but at the same time little was excreted unchanged in the urine. It may be that the drug is metabolized and excreted in a form that cannot be determined by the methods used here. The evidence from estimations of drug in chick tissues suggests that it is taken up and retained, particularly in liver and lung.

Attempt to Induce Drug Resistance to Compound 377C54 in P. gallinaceum

In none of the experiments described under "Methods" was the ED50 for 377C54 against the treated substrain appreciably different from that obtained against the normal strain. There was therefore no development of resistance.

Pharmacological Investigations

The LD50 of 377C54 given intravenously in mice was approximately 35 mg./kg. Marked respiratory distress was observed after 15 mg./kg. In a cat under chloralose anaesthesia, 3 mg./kg. had a mild hypotensive effect and 10 mg./kg. caused cardiac failure with a precipitous fall in blood pressure practically to zero and respiratory arrest. During artificial respiration, the blood pressure returned gradually to near the pre-injection value within 15 min. The hypotensive effect of 377C54 was uninfluenced by ganglion blocking agents or by atropine.

The toxic oral dose of 377C54 in cats was about 400 to 600 mg./kg. Five of ten cats survived such amounts and two of them became blind. In these, ophthalmoscopic examination revealed marked constriction of retinal vessels.

Acute experiments showed the drug to have no important effect on the plain muscle of the ileum or uterus *in vitro*, but concentrations of 10^{-6} to 3×10^{-6} caused some inhibition of the peristaltic reflex. No specific antagonism of acetylcholine or histamine was found in guinea-pig ileum or cat blood pressure experiments, and, except after doses causing severe hypotension (10 mg./kg.), there was no interference with the blood pressure responses to vagal stimulation or $N^1:N^1$-dimethyl-N^2-phenylpiperazinium iodide, or with the response of the nictitating membrane to preganglionic nerve stimulation in an anaesthetized cat. A further indication of lack of ganglion blocking or anti-parasympathetic effects was the absence of a mydriatic effect with 25 mg./kg. 377C54 i.p. in mice. No antagonism of the hypertension caused by adrenaline or (−)-noradrenaline was observed on the cat blood pressure. A concentration of 10^{-6} did not antagonize spasm of the isolated rat uterus preparation elicited by 5-hydroxytryptamine. The duration of pentobarbitone sodium anaesthesia in mice was unaffected by 25 mg./kg., i.v., and the analgesic action was at most slight with 50 mg./kg., s.c., in rats.

The pharmacological investigations were carried out by Dr. Green, Dr. Udall, and Dr. White at the Wellcome Research Laboratories, Beckenham. The authors are indebted to Miss C. Reynolds for her assistance in the laboratory.

REFERENCES

Burke, W. J., Kolbezen, M. J., and Stephens, C. W. (1952). *J. Amer. chem. Soc.*, **74**, 3601.

Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M., and Russell, P. B. (1951). *Brit. J. Pharmacol.*, **6**, 185.

Rollo, I. M. (1952). *Trans. R. Soc. trop. Med. Hyg.*, **46**, 474.

Wiselogle, F. Y. (ed.) (1946). *A Survey of Antimalarial Drugs 1941–1954.* Ann Arbor, Michigan: J. W. Edwards.

Brit. J. Pharmacol. (1957), **12**, 176.

THE PHARMACOLOGICAL ACTIONS OF MUREXINE (UROCANYLCHOLINE)

BY

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Murexine or urocanylcholine is a naturally occurring choline ester of urocanic acid which was found in very large amounts in the hypobranchial body of *Murex trunculus* and other prosobranchiate molluscs. In vertebrates and invertebrates, it was found to possess marked neuromuscular blocking and nicotinic actions, but was almost devoid of muscarinic effects. The blocking action of murexine was considered, on the basis of experimental and clinical evidence, to be of the " depolarizing " type. It was weaker than, but qualitatively very similar to, that produced by suxamethonium. The nicotinic action of murexine was stronger than that of suxamethonium in both experimental animals and human beings.

Murexine or urocanylcholine (β-[imidazole-4(5)]-acryl-choline) is a naturally occurring choline ester found in the hypobranchial body of some prosobranchiate molluscs, which might use it for defence or, more likely, for procuring food.

Murexine was first isolated by Erspamer (1948); its chemical constitution was determined by Erspamer and Benati (1953a, 1953b); and the proposed formula was confirmed by synthesis (Pasini, Vercellone and Erspamer, 1952). Sufficient quantities of the substance were made which permitted extensive pharmacological studies and a preliminary clinical trial. The present paper gives an account of these studies.

METHODS AND MATERIALS

Murexine Samples.—Both natural and synthetic murexine chloride hydrochloride (mol. w., 296.2; 1 mg.=0.76 mg. base) were used throughout the experiments. The natural product was prepared as described by Erspamer and Benati (1953b). The synthetic product was obtained according to the procedure of Pasini, Vercellone and Erspamer (1952) which has recently been improved by Pasini (unpublished observation).

During the course of experiments carried out to improve the synthetic procedure, it was observed that different murexine samples, identical according to analysis for elements and paper-chromatographic characteristics, showed slightly different biological activity. This may perhaps be attributed to the presence of isomers or rearrangement derivatives of murexine. Here we wish only to point out that the activity of the synthetic murexine samples used in the present experiments differed from that of the natural product by less than 5%; thus the synthetic product was at least 95% pure.

Preparation of Hypobranchial Extracts.—The hypobranchial bodies (median zone), removed from the living animal (*Murex trunculus*, *Murex brandaris* and *Tritonalia erinacea*) after cautious rupture of the shell, were placed in 3 to 4 volumes of acetone. After standing for 24 hr., the solvent was poured off and filtered, and the tissue re-extracted with a further 3 volumes of 90% acetone. The combined filtrates were stored in the refrigerator until used. The solvent was then evaporated under reduced pressure at 45° C., and the liquid brought to the desired volume with distilled water.

The bioassay was carried out on the isolated frog rectus abdominis muscle suspended in a 10 ml. bath of frog Tyrode solution bubbled with air.

Pharmacological Methods.—The doses required to cause head-drop in 50% of rabbits and to paralyse 50% of dogs were determined after rapid intravenous injection.

The anaesthetics used were chloralose (70 mg./kg., i.v.), pentobarbitone (30 mg./kg., i.v.), Pernocton (70 mg./kg., i.m.) and urethane (1 to 1.3 g./kg., i.v.). Cats and dogs received intravenous injections or infusions into the femoral vein, rabbits into the ear marginal vein, rats and mice into the tail vein. Blood pressure was recorded from the femoral or carotid artery. Respiration was recorded by means of a tracheal cannula connected to a tambour. Adequate oxygenation of the blood during apnoea was maintained by a respiratory pump or by passing oxygen through a tracheal catheter.

Muscarine-like action was tested on the guinea-pig and rabbit intestine, on the rabbit atrium and on the isolated frog heart. The isolated frog rectus abdominis

muscle and the spinal cat prepared as described by Burn (1950) were used to test for nicotinic effects.

Other preparations employed in customary fashion were the leech dorsal muscle, the enucleated frog and octopod eye (the latter being kept in sea water) and the heart of *Helix pomatia*, suspended in the liquid recommended by Zettler and Schlosser (1954). The cat, dog and rabbit sciatic nerve gastrocnemius preparations were prepared as described by Burn (1950). Maximal stimuli (rectangular current pulses of 7 msec. duration) were applied to the sciatic nerve through a unipolar electrode at the rate of 5 or 10/min., and the contractions were recorded semi-isometrically.

The rat phrenic nerve-diaphragm preparation as described by Bülbring (1946) was used in a few experiments. The drug was left in contact with the preparation for 4 min.

Results

Occurrence and Distribution of Murexine

Table I shows the murexine content (expressed in terms of free base) in different areas of the hypobranchial body of *M. trunculus*, *M. brandaris* and *T. erinacea*. Only traces of murexine were found in other tissues.

TABLE I

THE MUREXINE CONTENT OF THE HYPOBRANCHIAL BODY

M indicates the median zone (MA, pars anterior; MP, pars posterior); B, the branchial zone; and R, the rectal zone of the hypobranchial body (see Erspamer and Dordoni, 1947). HB indicates the entire hypobranchial body. The numerals in parentheses give the number of specimens examined. The weights are expressed in mg./g. of fresh tissue.

Tissue		Dry Residue of Acetone Extract (mg./g.)	Murexine Content (mg./g.)
M. trunculus	MP (151)	114	46·0
" "	MA (124)	60	16·5
" "	B (132)	24	1·2
" "	R (242)	31	1·0
" "	MP (50)	—	33·0
" "	MA (50)	—	11·0
" "	M (3,350)	82	23·0
M. brandaris	M (22)	50	1·3
" "	MP (19)	—	0·1
T. erinacea	HB (23)	—	7·6

The results in Table I show: (a) that virtually all murexine in the hypobranchial body was contained in the median zone, and that it was most concentrated in the posterior part (75%) in *M. trunculus*, and in the anterior part (90%) in *M. brandaris*; and (b) that there were very conspicuous differences in the murexine content of the hypobranchial body of different *Murex* species. For *M. trunculus*, the dry residue of the acetone extract of the median zone may contain 35% of murexine, for *M. brandaris* only 2.6%. Since the weight of the median zone of a hypobranchial body of *M. trunculus* is approximately 0.1 to 0.15 g. (average of 3,350 specimens), it is easy to calculate that each *Murex* contains 2 to 4 mg. of murexine base.

Whittaker (1955) and Keyl and Whittaker (1955) have added to the list of murexine-yielding molluscs two North Atlantic species related to *Murex*, *Urosalpinx cinerea* and *Thais lapillus*. The concentration of the choline ester in the whole organism was 360 and 230 μg./g. fresh tissue, respectively. Welsh (1955) has tentatively identified as murexine one of the quaternary ammonium bases responsible for the paralysing action displayed by acetone extracts of the tentacles of certain jellyfishes and siphonophores.

Pharmacological Actions

General Effects.—The basic action of murexine in both vertebrates and invertebrates is to provoke a paralysis of the skeletal musculature. Death is caused by anoxia secondary to peripheral respiratory arrest. The heart continues to beat vigorously for several minutes after respiration has ceased.

It is not worth while describing in detail the pattern of the acute intoxication provoked by murexine in mammals. It corresponds to that described for other neuromuscular blocking agents, particularly decamethonium (Paton and Zaimis, 1949) and suxamethonium (Bovet and Bovet-Nitti, 1955). In the dog, for example, relaxation occurred within 1 min. of a single intravenous dose of 0.5 mg./kg. of murexine chloride hydrochloride, became maximal within 2 min. and disappeared within 4 to 7 min. Transient apnoea sometimes occurred at the time of maximal effect. Muscular relaxation of longer duration was easily obtained by repeated injections at appropriate intervals, or by intravenous infusion.

Particularly if murexine was administered too rapidly, neuromuscular depression was regularly preceded by transient stimulation, as shown by an increase in muscular tension and the appearance of muscular fasciculations. Respiratory movements, too, before being depressed by the drug, were powerfully stimulated for some seconds. At the time of maximal effect there was a conspicuous increase in salivary secretion, signs of increased lachrymal and bronchial secretion and, frequently, evacuation of urine and faeces.

When injected by the subcutaneous route in rabbits, rats and mice, about 5 to 8 times the intravenous dose was required for equal maximal effects and the paralysis did not appear for 10 to 20 min. and lasted for about 1 to 2 hr. Muscular paralysis and respiratory depression were preceded or accompanied by polypnoea, muscular twitches and fasciculations all over the body, salivary hypersecretion, intestinal borborygmi (in the rabbit) and defaecation.

TABLE II
COMPARISON OF THE DOSES OF MUREXINE WITH THOSE OF OTHER NEUROMUSCULAR BLOCKING AGENTS WHICH HAVE THE SAME PHARMACOLOGICAL ACTIVITY AND TOXICITY
Doses are given in mg./kg.

	Murexine (Chloride Hydrochloride)	Suxamethonium (Iodide)	Decamethonium (Iodide)	Gallamine (Iodide)	Tubocurarine (Chloride)
Mouse: LD50 by i.v. route	8·1–8·7	0·55–0·60	0·75–1·33	3·14	0·15–0·16
Rabbit: head drop, ED50	0·65	0·20–0·25	0·10–0·14	0·45	0·15–0·20
Dog: paralysing dose ED50	0·35	0·20	0·10	0·40	0·20
Man: mean clinical dose by single injection ..	1·0–1·2	0·50–1·0	0·04–0·1	1·2–2·0	0·10–0·25

When death occurred, terminal asphyxial convulsions sometimes appeared, but these were mild owing to the almost complete muscular paralysis. Muscular twitchings and fasciculations persisted for 10 to 15 min. after death.

The activity and toxicity of murexine is compared with that of other neuromuscular blocking agents (see Bovet and Bovet-Nitti, 1955) in Table II.

When given subcutaneously, the LD50 of murexine in mice is approximately 50 mg./kg.; when administered by mouth the drug is ineffective in doses up to 1 g./kg. Under artificial respiration dogs can resist more than 200 times the paralysing dose of murexine.

In birds murexine, like suxamethonium and decamethonium, provokes contracture (extension cramp of the legs, opisthotonos) instead of muscular paralysis. Myosis and evacuation of the bowels are sometimes observed at the same time. In pigeons the minimum active dose by the intravenous route is approximately 0.05 mg./kg., the lethal dose 0.2 to 0.3 mg./kg. Frogs and fishes are paralysed, like mammals. In fishes, the last muscles to be paralysed and the first to recover are the branchial ones.

The injection of 30 to 40 mg./kg. of murexine into the branchial heart of *Eledone moschata*, an octopod, is followed by a short period (1 to 2 min.) of stimulation and motor agitation with deep, forced respiratory movements. Soon afterwards, muscular weakness supervenes and the animal lies motionless on the bottom of the aquarium. At this time pupil becomes constricted and iris discoloured. Within 7 to 10 min., the pupils are maximally constricted and the eyeballs have almost entirely disappeared, as they have apparently been retracted and covered by the adjacent tissues. From the eye the depigmentation extends progressively all over the body, affecting first the tentacles and then the mantle. The musculature of the animal is flaccid and the tentacles hang inertly; respiratory movements cease completely within 10 min. Respiratory paralysis is followed by terminal asphyxial convulsions, reappearance of intense pigmentation and then, finally, by slow, paralytic emission of the ink and arrest of the branchial hearts (15 min.). A tenth of the above dose produces similar phenomena, which, however, are less severe and disappear completely within 1 or 2 hours.

Although not directly observed, it is highly probable that murexine is capable of paralysing other molluscs, such as small bivalve molluscs, which can in this way be opened and devoured by the *Murex*.

Neuromuscular Block

Cat Gastrocnemius.—A comparison of the paralysing action of different doses of murexine chloride hydrochloride on the cat, dog, and rabbit gastrocnemius is given in Table III.

The majority of our experiments were carried out in cats anaesthetized with chloralose. In some experiments in which pentobarbitone was used, murexine appeared to be somewhat less effective. Indeed, after administration of 200 μg./kg. of the drug, the twitch depression was 40%, as compared with 74% in cats anaesthetized with chloralose; after 300 μg./kg. the twitch depression was 70% and 96% respectively.

As with all other blocking agents, the repeated administration of murexine at brief intervals resulted in drug cumulation. Whereas, for example, a first injection of 100 μg./kg. of murexine produced in one experiment a maximal twitch reduction of 25%

TABLE III
THE PARALYSING ACTION OF MUREXINE CHLORIDE HYDROCHLORIDE ON THE SCIATIC NERVE-GASTROCNEMIUS MUSCLE PREPARATION OF DIFFERENT SPECIES
The results are the mean of 3 to 10 experiments. M.t.r. = maximum twitch reduction (% of normal twitch); t = time required for 50% recovery.

Murexine (μg./kg.)	Cat Gastrocnemius		Dog Gastrocnemius		Rabbit Gastrocnemius	
	M.t.r.	t (min. and sec.)	M.t.r.	t (min.)	M.t.r.	t (min.)
100	31	3	0	—	0	—
200	74	3 45	53	6	0	—
250	92	8	—	—	0	—
300	96	8 50	78	7	0	—
500	100	15	—	—	65	6
1,000	100	32 48	92	15	80	11
2,000	100	—	100	30	—	—

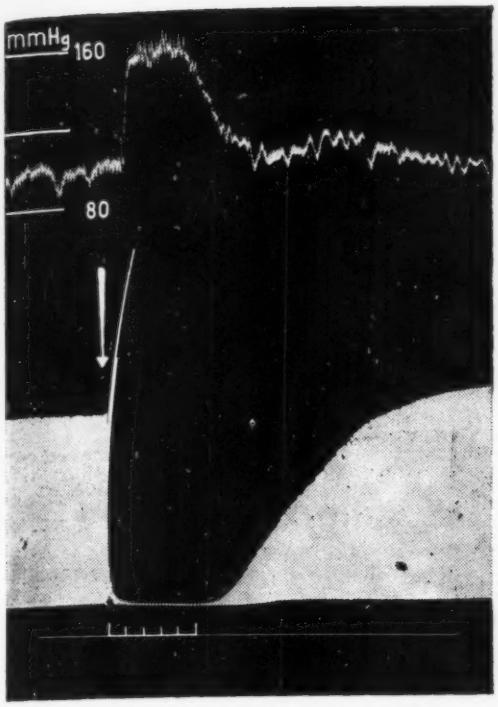

FIG. 1.—Cat sciatic nerve-gastrocnemius muscle preparation. Upper trace: blood pressure. Lower trace: muscle twitches in response to single maximal stimuli applied to the sciatic nerve. At the arrow murexine (300 μg./kg., i.v.). Neuromuscular block was preceded by an increased twitch height and a rise in blood pressure. Time, 1 min.

and required 3 min. for 50% recovery, a second injection, given 15 min. later, caused a maximum depression of 75%, and 50% recovery took 5 min.

Following the injection of small doses of murexine (250 to 300 μg./kg.), the twitch reduction was often

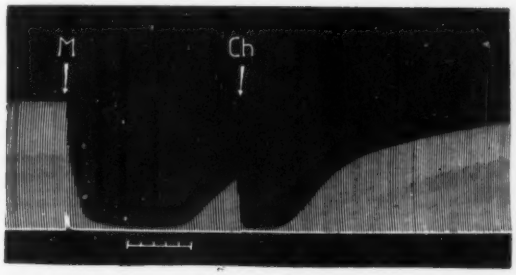

FIG. 2.—Cat sciatic nerve-gastrocnemius preparation. M, 300 μg./kg. murexine chloride hydrochloride. Ch, 5 mg./kg. choline chloride. Time, 1 min. Both injections were given intravenously. Choline, administered during the recovery phase following murexine paralysis, promptly caused further almost complete block.

preceded by a short-lived increase in the tension of the muscles as shown by the potentiation of some twitches (Fig. 1) and, between the contractions, fasciculations of the muscle could be seen. Another potentiation of the twitches was occasionally observed at the end of murexine paralysis.

5 mg./kg. of choline chloride, given intravenously during the recovery phase subsequent to 300 μg./kg. of murexine, promptly caused a renewed block (Fig. 2). A similar phenomenon was noted by Osterloch (1954) for suxamethonium. Eserine salicylate, intravenously in a dose of 50 μg./kg., did not affect appreciably the intensity of neuromuscular paralysis, but apparently prolonged it from 4 to 7 min. following 100 μg./kg. of murexine.

The intravenous infusion of 16 to 18 μg./kg./min. of murexine produced a gradually increasing twitch depression which began in 8 to 11 min., was 75% after 15 to 28 min., and was 95% to 100% after 30 to 60 min. A complete paralysis was then main-

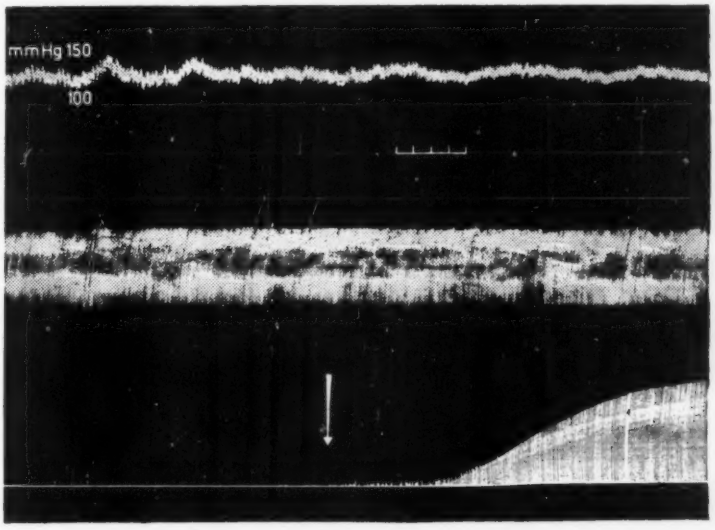

FIG. 3.—Cat anaesthetized with chloralose. Upper trace, blood pressure. Middle trace, respiration. Lower trace, twitches of the gastrocnemius muscle in response to single maximal stimuli to the sciatic nerve. Before the tracing commences, an intravenous infusion of murexine (17 μg./kg./min.) was started. At the arrow it was stopped. Time, 2 min. In spite of complete neuromuscular block, there was no change in blood pressure and no impairment of respiration.

tained as long as the infusion lasted. After the administration of the drug had been discontinued, a 25% recovery occurred within 8 to 20 min. (Fig. 3). With the same dose of suxamethonium both 100% twitch depression and complete recovery were attained more rapidly.

The intravenous injection of 5 mg./kg. of choline chloride, given during a murexine infusion when the twitch reduction was only 35%, immediately increased it to 90%. Tubocurarine proved to be a powerful antagonist to murexine, as it is for decamethonium and suxamethonium. Intravenous administration of 70 μg./kg. of tubocurarine completely abolished the paralysing action of a murexine infusion (Fig. 4). According to Philippot and Dallemagne (1956), 5-hydroxytryptamine partially

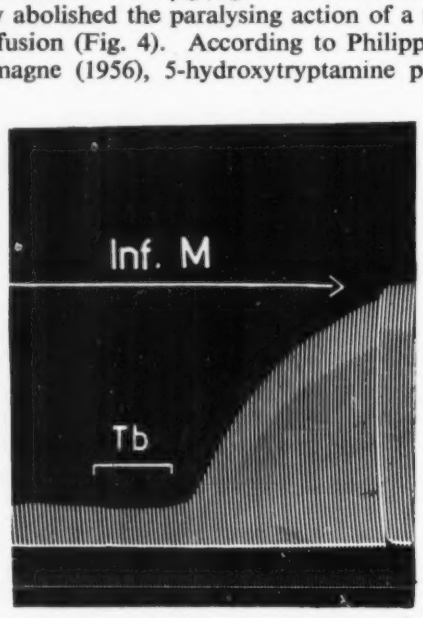

FIG. 4.—Muscle twitch as in Fig. 2. For the duration of the uppermost horizontal white line, an intravenous infusion of murexine (15 μg./kg./min.) was given (Inf. M.). The intravenous injection, during the period indicated by Tb, of tubocurarine (70 μg./kg.) antagonized the neuromuscular block due to murexine.

antagonizes the neuromuscular block caused by tubocurarine, whereas it has no effect on decamethonium paralysis. In our experiments, 25 to 50 μg./kg. of 5-hydroxytryptamine given intravenously did not affect the course of paralysis induced by murexine infusion.

In view of the close chemical and pharmacological similarity existing between murexine and suxamethonium, a comparison of the neuromuscular blocking action of the two drugs was carried out on the cat's gastrocnemius muscle preparation.

It is evident from the results shown in Table IV that suxamethonium is approximately 5 times as potent as murexine. The duration of the neuromuscular block is, however, longer for murexine.

TABLE IV

COMPARISON OF THE PARALYSING DOSES OF MUREXINE CHLORIDE HYDROCHLORIDE AND SUXAMETHONIUM CHLORIDE ADMINISTERED INTRAVENOUSLY IN THE CAT

No. of Expts.	Murexine (mg./kg.)	Suxamethonium (mg./kg.)	Maximum Twitch Reduction (% of Normal)	Time Required for 50% Recovery (min.)
8	0·1	—	31	3
3	—	0·025	48	2·33
10	0·2	—	74	3·75
5	—	0·05	91	4
3	0·25	—	92	8
3	—	0·1	100	7
8	0·3	—	96	9
2	—	0·2	100	8·5
2	0·5	—	100	15
2	—	0·5	100	12
5	1·0	—	100	33
2	—	1·0	100	14

It can be seen for example that, whereas after 1 mg./kg. of suxamethonium a 50% recovery can already be observed after 14 min., the same recovery after 1 mg./kg. of murexine takes place only after 33 min.

Dog and Rabbit Gastrocnemius.—Neuromuscular transmission in the dog was less sensitive to murexine than that in the cat, while that in the rabbit was still less sensitive. This example of variation of potency in different animal species can be seen in Table III.

Rat Diaphragm.—The preparation was not particularly sensitive to murexine. The minimum active dose is 15 to 20 μg./ml. Tubocurarine 0.5 μg./ml. almost completely antagonizes the twitch reduction produced by 25 μg./ml. of murexine. On the rat diaphragm, murexine is 2 to 3 times less potent than suxamethonium.

Blood Pressure

Cat.—In the intact animal, anaesthetized with chloralose or with pentobarbitone, the blood pressure response to rapid intravenous injections of murexine was rather variable. Small doses (20 to 50 μg./kg.) usually provoked a slight but transient hypotension; moderate doses caused transient hypotension (50 to 70 mm. Hg), transient hypertension (20 to 80 mm. Hg) (Fig. 1) or biphasic reactions (a rise followed by a fall of blood pressure). The injection of very high doses of murexine (5 to 10 mg./kg.) was generally followed by a sharp rise in blood pressure with subsequent fall, lasting for 10 to 15 min. The response often decreased progressively as doses were repeated. During intravenous infusion of murexine, at 15 to 30 μg./kg./min., which caused a complete muscular block, blood pressure changes were negligible (Fig. 3).

In contrast to the intact animal, the spinal cat always responded to intravenous murexine (0.2 to 3 mg./kg.) with a pure rise of blood pressure. In some cats this was proportional to the dose administered, while in others subsequent doses elicited decreasing reactions. Hexamethonium (2 to 5 mg./kg., i.v.) and Prosympal (diethylaminomethylbenzodioxan, 5 mg./kg., i.v.) reduce or abolish the murexine hypertension (Fig. 5). From this antagonism, from the characteristic form of the pressure curve and from the initial tachycardia it may be inferred that murexine hypertension is due to liberation of catecholamines in consequence of sympathetic ganglionic stimulation and/or suprarenal discharge; in fact it is a typical nicotinic effect of the substance.

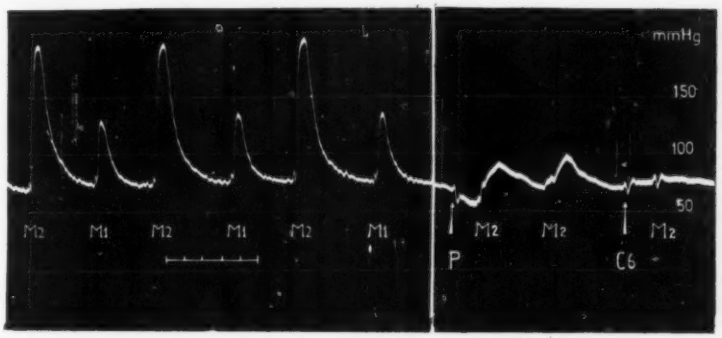

FIG. 5.—Blood pressure of a spinal cat. The pressure rise caused by murexine in this experiment was proportional to the dose. M1 and M2. 1 and 2 mg./kg. murexine chloride hydrochloride respectively. The blood pressure response to murexine was reduced by 5 mg./kg. of Prosympal (P), and was abolished by 5 mg./kg. of hexamethonium (C6). Time, 1 min.

Rabbit.—Doses of murexine which neither affected respiration (25 to 100 μg./kg.) nor caused hyperpnoea (100 to 500 μg./kg.) regularly provoked in the unanaesthetized rabbit transient and moderate hypotension (20 to 50 mm. Hg) which was barely proportional to the dose. With larger amounts of the drug, which produced respiratory arrest, the pressure response was often polyphasic (hypotension, hypertension, hypotension). If apnoea was completely avoided by the introduction of artificial respiration, there was no hypertension, and the fall of blood pressure, too, was conspicuously reduced. Hexamethonium (2 to 5 mg./kg.) had no effect on the murexine hypotension.

The behaviour of rabbits anaesthetized with urethane, and with urethane and pentobarbitone, was very similar to that observed in the unanaesthetized animal.

Dog.—Intravenous doses of 100 to 200 μg./kg. of murexine or more caused, in the animal anaesthetized with pentobarbitone or chloralose, an abrupt increase in the blood pressure. In some dogs the hypertension was roughly proportional both in intensity and duration to the doses of murexine, but in others there was tachyphylaxis. If hypertension was particularly intense and prolonged, it might be followed by a secondary fall of blood pressure. In one experiment, the intravenous injection of 25 mg./kg. of murexine caused a great increase in blood pressure, which rose from 140 to 280 mm. Hg and was followed by a fall to 105 mm. Hg lasting 40 min. As in the cat, murexine hypertension was opposed by hexamethonium (5 mg./kg.).

Heart

The isolated rabbit atrium was not affected by murexine chloride hydrochloride in concentrations up to 150 μg./ml. in nutrient liquid. The isolated frog heart responded to murexine concentrations of 1/1,000 to 1/3,000 with a moderate but transient decrease in the amplitude of beat. The effect was resistant to atropine, but promptly disappeared upon washing with fresh Tyrode solution.

On the isolated heart of *Helix pomatia*, murexine produced a negative chronotropic effect in a concentration of 10 μg./ml. in the nutrient liquid. Higher concentrations (30 to 40 μg./ml.) also reduced the amplitude of the beat or provoked (100 μg./ml.) a transient diastolic arrest followed by complete recovery.

In the intact anaesthetized dog, murexine in doses of up to 1 to 2 mg./kg. did not produce specific electrocardiographic changes other than a compensatory bradycardia with occasional manifestations of ventricular escape.

Respiration

Cat.—In the cat anaesthetized with chloralose or pentobarbitone, the minimum dose of murexine which affected respiration was 50 to 100 μg./kg. The usual response was a transient depression, followed by a period of respiratory stimulation; more rarely a short-lived stimulation was the only effect. It was not possible to correlate the respiratory changes induced by the drug with its effects on the blood pressure.

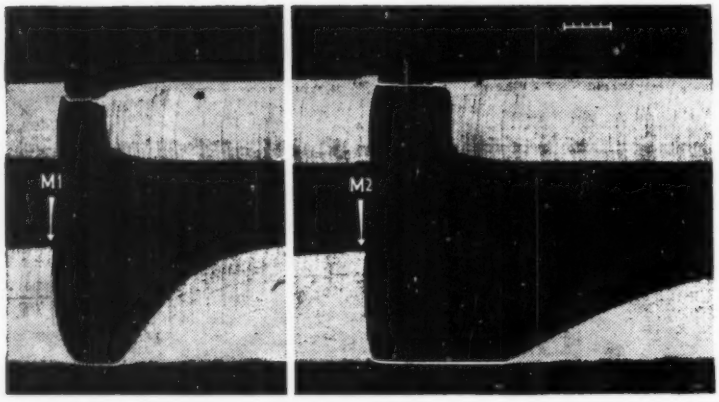

FIG. 6.—Cat anaesthetized with chloralose. Upper trace, respiration. Lower trace, contractions of the gastrocnemius muscle. After murexine injections (M1, 500 μg./kg.; M2, 1,000 μg./kg.) oxygen was administered through a tracheal catheter. Time, 1 min. The recovery of respiratory musculature was more prompt than that of the gastrocnemius muscle.

Higher doses of murexine (500 to 1,000 μg./kg.) regularly provoked respiratory arrest due to paralysis of the skeletal musculature. If oxygen was administered through a catheter introduced into the trachea, it was readily observed that recovery of respiratory muscles preceded that of other skeletal muscles (Fig. 6).

Rabbit.—In the unanaesthetized animal, doses of 25 to 50 μg./kg. did not affect respiration. Higher doses (100 to 500 μg./kg.) regularly increased the respiratory rate and volume. Doses of 1 mg./kg. and more caused respiratory arrest. Paralysis was sometimes followed by a period of hyperpnoea.

The behaviour of rabbits anaesthetized with urethane or pentobarbitone was similar, but animals anaesthetized with urethane and morphine always responded to murexine with a respiratory depression which lasted for several minutes. When murexine stimulated respiration, there was no correlation between intensity and duration of the moderate hypotension produced by the drug and the intensity and duration of hyperpnoea. It seems therefore probable that this was a nicotinic effect of murexine.

Dog.—The minimum dose of murexine active on respiration was 150 to 200 μg./kg. There was a very short-lived intense stimulation of respiratory movements, followed by depression or, according to the dose, by respiratory arrest. The changes were very similar in normal dogs and in animals anaesthetized with chloralose or pentobarbitone.

Smooth Muscle

Intestine.—The minimum dose of murexine which caused stimulation of the guinea-pig small intestine was approximately 5 to 10 μg./ml. There was no constant dose-response relationship. Both atropine 10^{-7} and hexamethonium 10^{-4} reduced the spasmogenic effect of murexine.

The rabbit small intestine was rather insensitive to murexine, and the weak spasm provoked was very irregular and not proportional to the dose. Acetylcholine was at least 5,000 times as potent as murexine.

On the rabbit large intestine, doses of 10 to 15 μg./ml. of murexine, or above, regularly produced a decrease in tonus which was sometimes proportional to the drug concentration. Hexamethonium 10^{-4} strongly reduced this adrenaline-like effect.

The results obtained on the intestinal smooth muscle demonstrated, in full accordance with those obtained on the isolated heart, that murexine completely lacked muscarinic effects.

Iris Muscle.—If murexine chloride hydrochloride was added to sea water in which the enucleated eye of an octopod (*Eledone moschata*, *Octopus vulgaris*) was immersed, there was a mydriasis and an expansion of the iris chromatophores (Fig. 7). The effect began to appear with a drug concentration of 1/20,000 to 1/50,000 and was very intense with 1/1,000 murexine. A maximal mydriasis was attained within 20 to 30 sec., and expansion of the chromatophores, preceded by alternate movements of expansion and retraction, was very conspicuous.

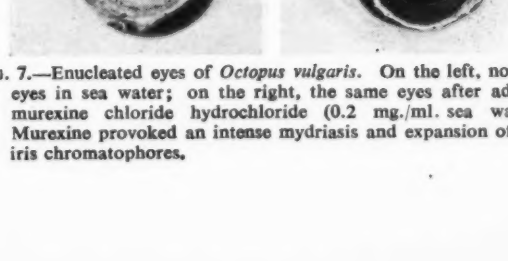

FIG. 7.—Enucleated eyes of *Octopus vulgaris*. On the left, normal eyes in sea water; on the right, the same eyes after adding murexine chloride hydrochloride (0.2 mg./ml. sea water). Murexine provoked an intense mydriasis and expansion of the iris chromatophores.

The toad and the frog enucleated eyes responded to murexine, 1/5,000 being required to produce a mydriasis.

D'Ermo (1947) has shown that crude extracts of the hypobranchial body of *M. trunculus* exerted a potent spasmogenic action on the isolated sphincter muscle of the horse iris. There was little doubt that this action was due to murexine which was active in a concentration of about 1/300,000.

Other Tissues

Frog Rectus Abdominis.—In our first study (Erspamer and Dordoni, 1947) murexine was found to induce a contracture in the frog rectus. This response was very similar to that produced by the methonium compounds and by suxamethonium, but differed from that provoked by acetylcholine, which had a slower onset and did not reach a plateau so readily. The relative potencies in stimulating the frog rectus were murexine chloride hydrochloride, 100; decamethonium iodide, 50; suxamethonium chloride, 180 to 200; acetylcholine bromide, 1,000.

Eserine salicylate 10^{-5} potentiated the response to murexine only by about 40%. Tubocurarine showed a strong antagonistic action; after 0.3 μg./ml. and 1.5 μg./ml. the contracture caused by 0.3 μg./ml. of murexine was reduced by 80 and 90% of the control respectively.

Leech Muscle.—Murexine causes a contracture in this preparation which closely resembled that produced by acetylcholine. Eserine salicylate 5×10^{-6} potentiated the response to murexine by about 2 to 4 times and that to acetylcholine by 1 to 2,000 times.

Isolated Cervical Ganglion of the Cat.—Kewitz (1955) found that the isolated superior cervical ganglion of the cat is stimulated by murexine, when the drug is added to the perfusion liquid. The action, which was as intense as that caused by acetylcholine, was reduced by adrenaline but not by scopolamine.

Isolated Brain Preparation of the Cat.—The intravenous injection of murexine, in doses of up to 10 mg./kg., did not produce any appreciable change in the electroencephalographic record.

Clinical Trial of Murexine

A preliminary clinical trial of murexine was carried out by Deblasi and Leone (1955) on 47 patients and by Ciocatto, Cattaneo and Fava (1956) on 123 patients. Murexine proved to be an interesting short-lasting muscle relaxant, worthy of a more extensive clinical investigation. The mean paralysing dose in adult patients was approximately 1 to 1.2 mg./kg., intravenously. Maximum effects were attained in 45 to 60 sec., and, following a single intravenous dose, the paralysis lasted 3 to 6 min. A long-lasting, satisfactory muscular relaxation could be obtained by slow intravenous infusion of a 1/1,000 solution of murexine in physiological saline.

Murexine caused several side-effects, which were mainly attributable to the nicotinic actions of the drug. Muscular paralysis was always preceded by and followed by fasciculations of the musculature; copious salivation was very common but was easily blocked by atropine; a moderate rise in blood pressure (10 to 15 mm. Hg) was frequent, but unimportant. Persistence of pharyngeal and laryngeal reflexes, lachrymation and sweating were rarely observed.

Table V summarizes the relative potency, rapidity of onset and duration of action of murexine and other curarizing agents in man (Ciocatto, Cattaneo and Fava, 1956). The potency of tubocurarine is arbitrarily taken as 100.

TABLE V

ACTIVITY OF MUREXINE IN MAN AS COMPARED WITH THAT OF OTHER MUSCLE RELAXANTS

The potencies are related to that of tubocurarine arbitrarily taken as 100.

	Blocking Potency	Time for Appearance of Maximum Effect (min.)	Duration of Action (min.)
Tubocurarine ..	100	1–2	25–30
Gallamine ..	21	1–2	20–25
Decamethonium	578	1–2	20–25
Suxamethonium..	34	½–¾	3–5
Murexine ..	20	¾–1	3–6

DISCUSSION

Murexine is one of the first neuromuscular blocking agents of animal origin to be chemically defined. It is highly probable that systematic studies in the field of invertebrates will lead to the discovery of the existence of murexine or closely related substances in other invertebrates, for defence or for securing food.

The materials available to *Murex* for the biosynthesis of murexine are common in the living organism, being choline and urocanic acid or some precursors of it, probably derived from the metabolism of histidine. It would certainly be worth while investigating in detail the processes involved in the synthesis of this new choline ester, particularly in view of the idea that similar processes may take place in the central nervous system of vertebrates (Gruner and Kewitz, 1955).

Murexine possesses two of the three types of pharmacological action seen in choline and its

derivatives, namely the nicotinic and the neuromuscular blocking actions. The muscarinic action, if any exists, is very weak. The relation between the chemical structure and the pharmacological actions of murexine derivatives will be discussed in greater detail elsewhere, but in connexion with the present work it is interesting to note the following: (*a*) Hydrogenation of the acrylic lateral chain into propionic caused a powerful increase in both nicotinic and neuromuscular blocking actions. The blocking effect of dihydromurexine on the cat gastrocnemius preparation was 4 to 5 times as great as that of murexine, and the nicotinic action on the frog rectus was 7 times as great. (*b*) The length of the lateral chain of the imidazole acid was of critical importance for the pharmacological effects. A three carbon atom lateral chain seemed to be optimal both for maximal blocking and nicotinic potency. Imidazoleacetyl- and imidazolecarboxylcholine on the one hand, and imidazolebutyrylcholine on the other, are less potent than imidazolepropionyl-choline. (*c*) Substitution of the ester linkage by an ether linkage reduced the blocking potency of dihydromurexine by about 6 times on the cat gastrocnemius, but considerably prolonged this action, and the nicotinic effect on the frog rectus was reduced by about 250 times. (*d*) Glässer and Pasini (1957) found that murexine chloride hydrochloride was twice as potent, on the cat gastrocnemius preparation and the frog rectus, as murexine bromide. The explanation of this surprising fact remains obscure.

In the absence of direct evidence, all the available experimental and clinical evidence suggests that murexine should be listed among the blocking agents acting by depolarization. Table VI shows the differences and similarities between tubocurarine (an example of the " competitive " blocking agents or " pachycurares "), suxamethonium choline (an example of the " depolarizing " blocking agents or " leptocurares ") and murexine.

TABLE VI

COMPARISON OF DIFFERENCES AND SIMILARITIES BETWEEN TUBOCURARINE, SUXAMETHONIUM AND MUREXINE

NMB = Neuromuscular block.

	Tubocurarine	Suxamethonium	Murexine
Action in mammals	NMB	NMB preceded by muscular fasciculation	NMB preceded by muscular fasciculation
„ „ birds ..	„	Nicotinic contraction	Nicotinic contraction
Effect of anticholinesterases on NMB	Antagonism	No antagonism	No antagonism
Effect of tubocurarine on NMB	Potentiation	Antagonism	Antagonism
Action on frog rectus	No contracture	Contracture	Contracture

Among the known " depolarizing " agents, the substance most closely related to murexine is suxamethonium. The blocking potency of murexine in intact dogs and human beings was approximately 50% to 60% and in the cat was about 20% of that of suxamethonium. As with gallamine, the acute toxicity of murexine was surprisingly low in mice, being about 13 to 15 times lower than that of suxamethonium. Murexine was considerably more potent than suxamethonium in its nicotinic actions on the blood pressure of mammals, and in its power to produce muscular fasciculations which precede paralysis. Apart, however, from the above quantitative differences, murexine behaves very similarly to suxamethonium and, like the latter, it is characterized by the rapid onset and short duration of its pharmacological effects.

In preliminary clinical trial as an adjuvant in anaesthesia murexine gave a good neuromuscular blocking effect, accompanied by marked nicotinic side-effects, some of which were easily counteracted by atropine, while others apparently cannot be avoided. More extensive clinical investigation is necessary to establish whether murexine can be employed as a safe and valuable muscle-relaxant drug.

REFERENCES

Bovet, D., and Bovet-Nitti, F. (1955). *Scientia med. italica*, **3**, 484.

Bülbring, E. (1946). *Brit. J. Pharmacol.*, **1**, 38.

Burn, J. H. (1950). *Biological Standardization*, pp. 347 and 351. Oxford: Oxford University Press.

Ciocatto, E., Cattaneo, A., and Fava, E. (1956). *Mi nerva anestes.*, **22**, 197.

Deblasi, S., and Leone, U. (1955). Ibid., **21**, 137.

D'Ermo, F. (1947). *Boll. Oculistica*, **26**, 763.

Erspamer, V. (1948). *Experientia*, **4**, 226.

—— and Benati, O. (1953a). *Science*, **117**, 161.

—— —— (1953b). *Biochem. Z.*, **324**, 66.

—— and Dordoni, F. (1947). *Arch. int. Pharmacodyn.*, **74**, 263.

Glässer, A., and Pasini, C. (1957). *Experientia*, in the press.

Gruner, G., and Kewitz, H. (1955). *Naturwissenschaften*, **42**, 628.

Kewitz, H. (1955). *Arch. exp. Path. Pharmak.*, **225**, 111.

Keyl, J., and Whittaker, V. P. (1955). *Amer. J. Physiol.*, **183**, 634.

Osterloch, G. (1954). *Arch. exp. Path. Pharmak.*, **222**, 360.

Pasini, C., Vercellone, A., and Erspamer, V. (1952). *Ann. Chem.*, **578**, 6.

Paton, W. D. M., and Zaimis, E. J. (1949). *Brit. J. Pharmacol.*, **4**, 381.

Philippot, E., and Dallemagne, M. J. (1956). *Arch. int. Pharmacodyn.*, **105**, 426.

Welsh, J. H. (1955). *Deep Sea Research*, **3**, Suppl., 287.

Whittaker, V. P. (1955). *3ème Congrès internat. Biochimie, Bruxelles.* Rés. des Communicat., p. 31.

Zettler, G., and Schlosser, L. (1954). *Arch. exp. Path. Pharmak.*, **222**, 345.

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THE TREATMENT OF EXPERIMENTAL TOXOPLASMOSIS IN RABBITS

BY

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Sulphadimidine alone is of little value in treating experimental toxoplasmosis in rabbits, because most rabbits acetylate the drug very rapidly. Within a short time of dosing such animals, there is little or none of the uncombined drug in the blood. In rabbits which do not acetylate sulphadimidine rapidly, relatively high concentrations of the free sulphonamide can be attained in the blood: in such animals, toxoplasmosis responds to treatment with sulphadimidine. Sulphathiazole is not acetylated so rapidly and is effective even in rabbits which acetylate sulphadimidine quickly. Pyrimethamine, even in doses as high as 50 mg. three times daily, is ineffective; dapsone is effective.

There are few reports upon the action of sulphonamides in the treatment of toxoplasmosis in rabbits. Good results have been reported with sulphathiazole (Sabin and Warren, 1942; Biocca, 1945); toxoplasmosis of the uveal tract in rabbits was found to respond with moderate success to treatment with dapsone, and cures were obtained with a combination of dapsone and cortisone (Beverley, Beattie and Fry, 1954). We have now compared the efficiency of sulphadimidine with that of pyrimethamine and of dapsone in experimental toxoplasmosis in rabbits, and have investigated the way in which the therapeutic effect is influenced by the ability of individual rabbits to acetylate sulphonamides.

METHODS

Comparison of the Efficiency of Sulphadimidine, Dapsone, and Pyrimethamine.—Twenty-seven rabbits, which had been shown to have no detectable complement-fixing antibodies to toxoplasma, were arranged in groups of 3, each group containing rabbits weighing 2.0, 2.5, and 3.0 kg. The rabbits were inoculated intradermally with 0.2 ml. of diluted peritoneal exudate taken from a mouse infected 3 days earlier with the *RH* strain of *Toxoplasma gondii*. The inoculum contained about 80,000 organisms.

Beginning 2 hr. after inoculation, each rabbit was treated 3 times daily for 9 days with sulphadimidine, dapsone, or pyrimethamine. Sulphadimidine was given in doses of 50, 100, or 200 mg./rabbit, dapsone in doses of 25, 50, or 100 mg./rabbit, and pyrimethamine in doses of 12.5, 25, or 50 mg./rabbit (Beverley *et al.*, 1954). Drugs were administered in gelatin capsules. The rabbits were fed on a pellet diet (Diet No. 18, Associated London Flour Millers).

Estimation of Sulphonamides.—Doses of drugs were given in gelatin capsules or suspended in 5 ml. of water and given by stomach tube under pentobarbitone (Nembutal) anaesthesia, or by intravenous injection of an aqueous solution of a soluble salt (*p*H adjusted to 7.0).

Blood was collected from an ear vein, heparinized, and 1 ml. samples transferred to centrifuge tubes containing 2 ml. of 0.2 M-Na_2HPO_4. Protein was precipitated by the addition of 1 ml. 20% w/v trichloracetic acid, and removed by centrifuging. Free and acetylated sulphonamides were determined colorimetrically by the procedure of Bratton and Marshall (1939), as modified by Francis and Spinks (1950). A Unicam spectrophotometer SP 500 was used for the measurement of absorption at 525 mμ.

RESULTS

All three rabbits which were given 100 mg. of dapsone and two of those receiving 50 mg. three times a day survived. Pyrimethamine, even in doses as high as 50 mg. thrice daily, gave no protection. Only two of the rabbits receiving sulphadimidine survived; one of these had been given 200 mg. and the other 50 mg. three times daily. Sulphadimidine was therefore not very active in controlling toxoplasmosis in rabbits, although it had proved more effective than dapsone in infected mice (Beverley and Fry, 1956, 1957).

Blood Concentrations of Sulphadimidine.—Two rabbits (Nos. 273 and 274) were each given three capsules containing sulphadimidine equivalent to a dose of 400 μg./kg. Both rabbits showed two maxima in the blood concentration of the sulphonamide, one at 1 hr. and the other at 5 hr. after

the dose. A single maximum was expected, and the observed result may have been due to the capsules failing to dissolve at the same time. The most striking feature of the results was that, whereas rabbit 274 reached a peak concentration of 170 μg. sulphadimidine/ml. of blood, the highest concentration in rabbit 273 was only 16 μg./ml. To ensure that all the drug was free in the intestine immediately after giving the dose, 200 mg. of the drug was given by oesophageal tube to the same two rabbits, anaesthetized with pentobarbitone given intravenously. Once again the level of sulphadimidine in 274 was much higher than in 273 (Fig. 1). These results led us to suspect that

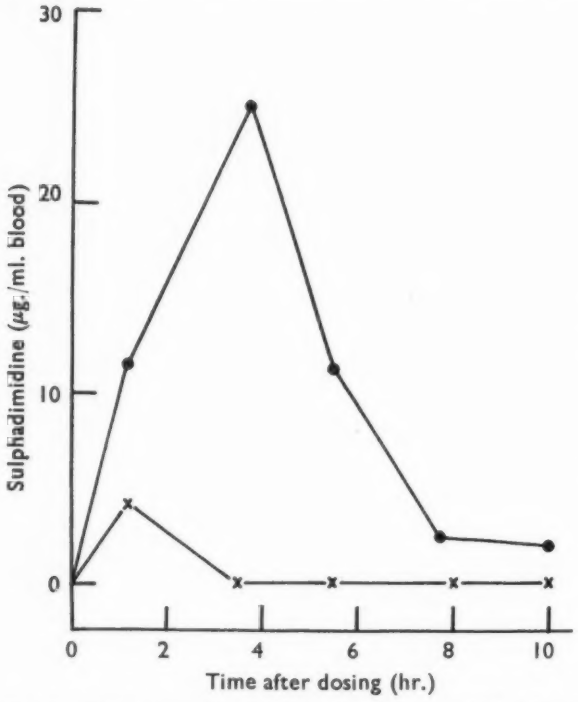

FIG. 1.—Blood concentration of sulphadimidine in rabbits after a single dose of 200 mg. given by oesophageal tube. Rabbits 273 (crosses) and 274 (dots) were anaesthetized with pentobarbitone.

the low activity of sulphadimidine against toxoplasma was due to the inability of those rabbits which died to attain a high enough concentration of the drug in the blood. The two survivors (263 and 267) from the groups of infected rabbits given sulphadimidine were each given one dose of 200 mg. sulphadimidine by oesophageal tube, and, whilst there was an appreciable difference between the two animals (Fig. 2), it was to be noted that rabbit 263 (crosses) with the lower blood concentration of sulphadimidine was the survivor which had received 200 mg. doses of sulphadimidine, whereas rabbit 267 (dots) with the higher concentration had received only 50 mg. doses.

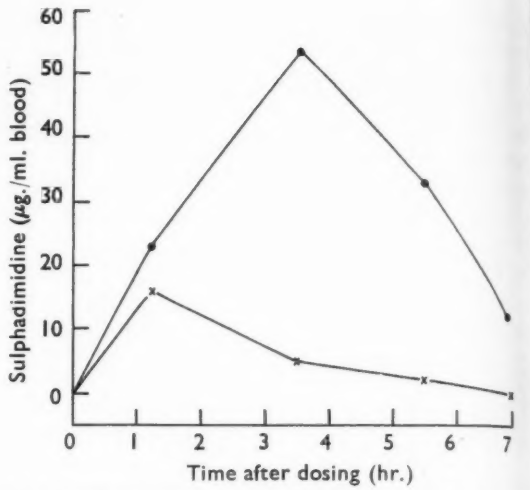

FIG. 2.—Blood concentration of sulphadimidine in two rabbits which were successfully treated with sulphadimidine after being infected with toxoplasma. Rabbits 263 (crosses) and 267 (dots) each received a single dose of 200 mg. sulphadimidine by oesophageal tube under pentobarbitone anaesthesia.

These preliminary experiments indicated that there might be considerable variation in the ability of rabbits to attain significant concentrations of sulphadimidine in their blood. The results of an experiment in which eleven rabbits were each given 200 mg. sulphadimidine by oesophageal tube supported this conclusion (Table I). In eight of the rabbits the blood concentrations of sulphadimidine were no more than 4 μg./ml. an hour after dosing, and only two had concentrations greater than 10 μg./ml. Such results might reflect differences in the ease with which sulphadimidine is absorbed by different rabbits. Four rabbits were, therefore, each given 200 mg.

TABLE I

BLOOD CONCENTRATION OF SULPHADIMIDINE IN RABBITS

Each rabbit was anaesthetized with pentobarbitone intravenously and given 200 mg. sulphadimidine by oesophageal tube. Blood samples taken at 1, 3, and 5 hr. after dosing.

Rabbit No.	Type	Weight (kg.)	Conc. of Sulphadimidine (μg./ml. Blood)		
			1 hr.	3 hr.	5 hr.
295	English	3·20	< 0·5	0	0
296	Sable	2·62	14·4	13·0	5·4
297	Dutch	1·76	1·8	2·8	0
298	English × Dutch	1·66	3·4	0	0
299	English	2·30	31·8	14·4	5·8
301	Agouti	3·80	0	0	0
302	,,	3·26	< 0·5	0	0
303	Dutch	1·60	4·0	2·2	0
304	Blue	2·50	< 0·5	0	0
305	English	3·00	1·0	< 0·5	< 0·5
306	Dutch	2·30	3·4	2·6	4·0

of sulphadimidine by oesophageal tube and then kept in metabolism cages. Analysis of the blood 2 hr. and 4 hr. after dosing showed that, whereas in rabbits 263 and 267 the blood concentration of the free sulphonamide was relatively high and there was only a small amount in the acetylated state, in rabbits 301 and 302 there was no free sulphadimidine although there were considerable amounts of the acetylated form (Table II). Analysis of the urine excreted within 24 to 30 hr. after

TABLE II

CONCENTRATION OF FREE AND ACETYLATED SULPHADIMIDINE IN BLOOD AND URINE OF RABBITS

Each rabbit was anaesthetized with pentobarbitone intravenously and given 200 mg. sulphadimidine by oesophageal tube. The animals were kept in metabolism cages and blood samples taken 2 and 4 hr. after dosing.

Rabbit No.	Blood				Urine				
	Sulphadimidine (μg./ml.)				Total Vol. (ml.)	Period (hr.)	Sulphadimidine		
	2 hr.		4 hr.				Free (μg./ml.)	Acetylated (μg./ml.)	Total (mg.)
	Free	Acetylated	Free	Acetylated					
263	37·1	6·1	28·0	5·3	133	24	274	721	132
267	46·1	3·8	27·5	4·8	156	21	276	616	139
301	0	28·5	0	21·3	188	30	9·5	761	145
302	0	29·1	0	19·7	205	30	3·8	406	84

dosing revealed that all the rabbits had absorbed at least 80 mg. (40% of the dose of sulphadimidine), and in three of them about 130 mg. (65% of the dose) had been absorbed. It was concluded that all four rabbits absorbed sulphadimidine easily and the differences in the blood concentration of free sulphadimidine were the outcome of the speed with which rabbits are able to

TABLE III

CONCENTRATION OF FREE AND ACETYLATED SULPHADIMIDINE IN BLOOD OF RABBITS AFTER INTRAVENOUS INJECTION OF THE DRUG

Each rabbit received 10 ml. of solution containing 1.2 mg./ml. over a period of 4 min.

Time after Dose (min.)	Sulphadimidine Conc. in Blood (μg./ml.)							
	Rabbit 263		Rabbit 267		Rabbit 295		Rabbit 301	
	Free	Acetylated	Free	Acetylated	Free	Acetylated	Free	Acetylated
0	0	0	0	0	0	0	0	0
7	10·1	0	10·5	0	4·9	4·6	3·7	4·5
20	7·9	0·7	8·2	1·3	1·9	5·2	1·5	5·6
40	5·4	0	5·2	0	0·8	3·1	0·6	3·3
70	4·2	0·5	4·1	1·1	0·6	2·2	0	3·7
120	2·1	0·7	1·9	0·2	—	—	—	—

acetylate the drug. This was confirmed by an experiment in which four rabbits were each injected intravenously with 12 mg. of sulphadimidine dissolved in water. In the blood of rabbits 263 and 267 described as "slow acetylaters" on the basis of the results of the previous experiments, there were at least 4 μg./ml. of uncombined sulphadimidine in the blood even 1 hr. after dosing, and little of the drug was acetylated (Table III). On the other hand, with rabbits 295 and 301, suspected to be "quick acetylaters," very little of the sulphadimidine in the blood was in the free state after the first 20 min.; most was in the acetylated form. The rapidity with which acetylation of sulphadimidine takes place in some rabbits was shown by giving 1 g. by oesophageal tube to rabbit 302. The maximal concentration of free sulphadimidine in the blood was only 3.4 μg./ml.; this concentration was maintained for about 5 hr. Within 1 hr. of dosing the blood contained 61 μg./ml. of acetylsulphadimidine.

Blood Concentrations of Sulphathiazole.—In early reports of the successful treatment of toxoplasmosis in rabbits the sulphonamide used was sulphathiazole (Sabin and Warren, 1942; Biocca, 1945). It was, therefore, decided to determine whether it is possible to obtain greater blood concentrations of sulphathiazole than of sulphadimidine. Two rabbits, 295 and 305, suspected to be "quick acetylaters" of sulphadimidine (Table I) were given 200 mg. sulphathiazole by mouth. The blood concentrations of sulphathiazole, although relatively low, were greater than those attained with sulphadimidine (Table IV).

TABLE IV

CONCENTRATION OF FREE AND ACETYLATED SULPHATHIAZOLE IN BLOOD OF RABBITS AFTER GIVING THE DRUG BY OESOPHAGEAL TUBE

Each rabbit was anaesthetized with pentobarbitone intravenously and given 200 mg. sulphathiazole.

Time after Dose (Hr.)	Sulphathiazole Conc. in Blood (μg./ml.)			
	Rabbit 295		Rabbit 305	
	Free	Acetylated	Free	Acetylated
1	12·5	20·3	5·9	16·8
3	2·7	10·1	2·4	6·4
5	1·3	2·7	1·1	1·6

Comparative Therapeutic Efficiency of Sulphathiazole and Sulphadimidine.—Three pairs of rabbits were inoculated intradermally with toxoplasma; one pair (301 and 302, "quick acetylaters") received sulphadimidine, another pair (295 and 305, "quick acetylaters") received sulphathiazole, and the remaining pair (296 and 299, "slow acetylaters") received sulphadimidine. The drugs were given in capsules (200 mg./rabbit) at four-hourly intervals (omitting one dose at 4 a.m. for nine days). On the eighth day rabbits 301 and 302 died. These were both "quick acetylaters" and known to be unable to attain significant concentrations of free sulphadimidine in the blood (Table I). The treatment of the other two pairs of rabbits was stopped on the

ninth day and they were still alive and well three weeks later. It was concluded that sulphathiazole is likely to be superior to sulphadimidine in the treatment of toxoplasmosis in rabbits. It appears from the results of these experiments that rabbits vary in their abilities to acetylate sulphonamides, and that different sulphonamides are acetylated to different extents in the same rabbit. To test this conclusion, doses of 200 mg. of sulphadiazine, sulphamerazine, and sulphanilamide were given in separate experiments by oesophageal tube to two rabbits. One (295) was a "quick acetylater" and the other (296) a "slow acetylater," on the basis of the experiments with sulphadimidine shown in Table I. Rabbit 295 acetylated sulphadiazine, sulphamerazine and sulphanilamide at a rapid rate (Table V) and it could be reasonably predicted that in this animal sulphamerazine and

TABLE V

CONCENTRATION OF FREE AND ACETYLATED SULPHADIAZINE, SULPHAMERAZINE, AND SULPHANILAMIDE IN THE BLOOD OF RABBITS

Each rabbit was anaesthetized with pentobarbitone intravenously and given 200 mg. of the sulphonamide by oesophageal tube.

Drug	Time after Dose (Hr.)	Sulphonamide Conc. in Blood (μg./ml.)			
		Rabbit 295		Rabbit 296	
		Free	Acetylated	Free	Acetylated
Sulphadiazine..	1	13·6	29·6	28·2	3·5
	3	14·7	20·2	44·7	5·9
	5	10·7	13·1	47·0	5·2
Sulphamerazine	1	3·5	28·6	18·4	5·6
	3	0·8	23·0	39·0	21·5
	5	2·7	35·5	35·2	18·2
Sulphanilamide	1	12·0	15·7	18·4	1·2
	3	3·7	16·6	28·6	3·4
	5	1·6	11·5	27·5	3·5

sulphadimidine would be of little therapeutic value against toxoplasma. On the other hand, the blood concentrations of sulphanilamide (Table V) and of sulphathiazole (Table IV) for at least an hour after dosing and of sulphadiazine for 5 hr. (Table V) were such that these drugs might be of some value in combating infection. In rabbit 296, a "slow acetylater" of sulphadimidine, high concentrations of free sulphadiazine, sulphamerazine and sulphanilamide were readily attained (Table V).

DISCUSSION

In the above experiments, the concentration of free sulphadimidine in the blood has been taken as an index of the amount of the drug available to combat infection. Sulphadimidine is successful in curing toxoplasmosis in mice, but its value in treating rabbits is doubtful because of the great variation in the concentration of free sulphadimidine which can be attained in the blood of these animals. The evidence presented in this paper supports the view that the wide range of blood concentrations of free sulphadimidine in rabbits is due to differences in the speed with which different rabbits can acetylate sulphadimidine and thus destroy its therapeutic properties. The enzymatic mechanism for the acetylation of sulphonamide has already been investigated in detail (Lipmann, 1945) and occurs, in the rabbit, only in the liver (van Winkle and Cutting, 1940). It will be of interest to establish whether or not this quantitative variation in the ability of rabbits to acetylate sulphadimidine can be related to differences in the amount of the enzyme system/unit weight of liver or to the size of the liver in proportion to the whole animal. Furthermore, the nutrition of the animal may affect its acetylating abilities, because coenzyme A is an important co-factor in the acetylation of sulphonamides and the amount of coenzyme A present in the liver will be related, in part at least, to the amounts of pantothenic acid present in the animal's diet. Sulphathiazole appears to be more effective than sulphadimidine in the treatment of experimental toxoplasmosis in rabbits: this is probably because it is not acetylated as quickly, and an effective therapeutic concentration of sulphathiazole can therefore be maintained for a longer period. Sulphanilamide, sulphathiazole, and sulphadiazine are already known to be acetylated at different speeds by the acetylase system of pigeon liver (Lipmann, 1945), and there is some evidence that the same applies in man (Janeway, 1942).

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REFERENCES

Beverley, J. K. A., Beattie, C. P., and Fry, B. A. (1954). *Brit. J. Ophthal.*, **38**, 489.
—— and Fry, B. A. (1956). *J. gen. Microbiol.*, **14**, ii.
—— —— (1957). *Brit. J. Pharmaco.*, **12**, 189.
Biocca, E. (1945). *Arch. Biol., S. Paulo*, **29**, 82.
Bratton, A. C., and Marshall, E. K., Jr. (1939). *J. biol. Chem.*, **128**, 537.
Eyles, D. E. (1953). *Amer. J. trop. Med. Hyg.*, **2**, 429.
—— and Coleman, N. (1953). Ibid., **2**, 54.
Francis, J., and Spinks, A. (1950). *Brit. J. Pharmacol.*, **5**, 565.
Janeway, C. A. (1942). *New Engl. J. Med.*, **227**, 989.
Lipmann, F. (1945). *J. biol. Chem.*, **160**, 173.
Sabin, A. B., and Warren, J. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 19.
Summers, W. A. (1949). *Amer. J. trop. Med.*, **29**, 889.
van Winkle, W., Jr., and Cutting, W. C. (1940). *J. Pharmacol.*, **69**, 40.

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SULPHADIMIDINE, PYRIMETHAMINE AND DAPSONE IN THE TREATMENT OF TOXOPLASMOSIS IN MICE

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Sulphadimidine, dapsone, and pyrimethamine have been tested alone and in various combinations for their therapeutic effect against toxoplasma infection in mice. In the treatment of active infection, sulphadimidine by itself was effective, but relapses were common. Pyrimethamine gave complete cures and prevented the carrier state when used in doses near to the toxic level. Dapsone alone was not as good as either of the other two drugs tested. The best combination was found to be sulphadimidine and pyrimethamine, which were synergic. In doses well below the toxic level, this combination not only controlled the acute infection but also prevented relapses and the development of the carrier state. Dapsone and pyrimethamine were also synergic, but were not as effective as the previous combination. No synergy was found between dapsone and sulphadimidine. The mechanism of relapse and the development of the carrier state and the modes of action of the drugs alone and in combination are discussed.

Warren and Sabin (1942) reported discrepancies between the *in vitro* and *in vivo* effects of drugs against *Toxoplasma gondii*. Most subsequent experiments on the action of drugs against this organism have therefore been done *in vivo*, chiefly with mice and rabbits. Antibiotics are almost without effect and the little action they have probably results from their influence on secondary infections. Fungicides and compounds containing arsenic and antimony are likewise ineffective; so are the majority of the antimalarial drugs, the exceptions being the sulphonamides and the 2:4-diaminopyrimidines. Sulphonamides have been shown to be toxoplasmicidal by many workers. Eyles and Coleman (1953a) showed that there was little to choose between the three pyrimidine sulphonamide derivatives, namely sulphadiazine, sulphamerazine and sulphadimidine, and that these compounds were superior to sulphathiazole, sulphapyridine and sulphanilamide. The diphenylsulphones were shown to be active by Summers (1949) and by Cross (1951). Eyles and Coleman (1952) showed that pyrimethamine (Daraprim) was the most active of a group of 2:4-diaminopyrimidines, and that the activity was enhanced by the concurrent administration of sulphadiazine (Eyles and Coleman, 1953b).

With mice as the test animals and using sulphadimidine, dapsone and pyrimethamine as representatives of the three groups of active drugs, we have attempted to assess (1) their relative value as short-term therapeutic agents and as substances which may prevent or eliminate the carrier state, (2) the ratio of effective dose to toxic dose, and (3) whether a synergy exists between pairs of the drugs. A preliminary account of our results has been given elsewhere (Beverley and Fry, 1956).

METHODS

Test Animals.—Mice were caged in groups of 6 and were allowed as much food and water as they wished. They were fed on a diet compressed into cubes. (Diet No. 86, North-Eastern Agricultural Co-operative Society Ltd., Aberdeen).

Inoculation.—The *RH* strain of toxoplasma (Sabin, 1941) was used. It had been maintained in our laboratory by twice-weekly mouse passage for the past six years. The inoculum consisted of peritoneal fluid from infected mice, diluted with saline so as to contain 15,000 to 20,000 extracellular toxoplasms in 0.013 ml. The number of extracellular toxoplasms was determined by counting in a Neubauer chamber. In order to simulate a natural infection, the mice were anaesthetized lightly with ether and one drop (0.013 ml.) of inoculum was allowed to fall from the tip of a 27 S.W.G. needle held 0.25 in. above the nostrils. Tests for potency showed such an inoculum to contain from 750 to 1,250 LD50 doses, determined by giving tenfold saline dilutions of the test dose to groups of 6 mice and calculating the titre by the methods of Reed and Muench (1938). In some of the experiments large numbers of mice, up to 300, were used. Even

though it was possible to inoculate all of them within 1 hr. it was thought that the virulence of the inoculum might lessen within that period. To determine if this was so, one set of control mice was inoculated before any of those to have treatment, a second set in the middle of those to be treated, and a third set at the end. No appreciable difference in survival time was found in these three groups in any of the experiments.

Treatment.—The drugs were given in the diet because it is the most practical way of treating large numbers of mice for a long period, and because mice eat at frequent intervals and a more uniform blood concentration can be maintained by this method than by oesophageal intubation. Drugs were added in the required amounts to 200 g. of powdered diet. After mixing, 100 ml. of water was added and the mixture worked into a doughy mass. This was recast into cubes by means of an apparatus designed to force the mixture through jets of suitable size. The wet cubes were dried in a well-ventilated oven at 56° C. overnight. Treatment started immediately after inoculation (unless otherwise stated) and was continued for 1, 2, 3, or 4 weeks.

Toxicity.—Drugs were fed in different concentrations in the diet of six-week-old mice for a fortnight. The toxic dose (TD) was taken to be that concentration which just prevented the mice from gaining weight.

Determination of Optimum Ratios of Drug Mixtures.—Various combinations of four different geometric fractions of the toxic dose of sulphadimidine and pyrimethamine were fed to 24 groups of mice. The survival times, relapse times, carrier states, and complete cures for each group were recorded. A similar experiment was made with mixtures of dapsone and pyrimethamine. The effective dose was taken as the lowest concentration of drug or drugs in the diet which, when given for 28 days, ensured that all mice so treated survived for 28 days ; these concentrations were expressed as fractions of the TD.

Assessment of Results.—An autopsy was done on each animal which died during the period of treatment or in the subsequent observation period of 4 weeks. Smears were made from the liver, lung, spleen, brain, and exudate (if present), and stained with methylene blue. In order to demonstrate the presence or absence of the carrier state, all animals which survived the observation period were killed by dislocation of the neck. The whole of the brain, half the spleen, and a portion of the liver about 0.75 in. square from the anterior edge were removed and ground with 2.5 ml. of saline containing 1,000 i.u. of penicillin and 100 i.u. of streptomycin/ml. After standing for ½ hr., the supernatant fluid and as many of the finer pieces of tissue as possible were aspirated into a syringe through a 20 S.W.G. needle. The fluid thus collected usually amounted to 2.0 ml., and one-half of it was inoculated intraperitoneally into each of 2 mice. If toxoplasms were present the inoculated mice died in 9 to 11 days. In early experiments a second passage was made from the mice which survived the first ; this procedure failed to reveal any further carriers.

Concentrations of Drugs.—Groups of 6 mice were fed for 3 days on a diet containing 1/2 TD of each of the drugs, and then bled under ether anaesthesia. The blood levels of sulphonamide and sulphone were estimated by the method of Bratton and Marshall (1939), as modified by Francis and Spinks (1950).

RESULTS

Toxicity.—The toxic doses were for sulphadimidine, 1.6 g./100 g. diet ; for dapsone, 200 mg./100 g. diet ; and for pyrimethamine, 50 mg./100 g. diet.

Duration of Treatment.—Four groups of 6 mice were treated with 1/2 TD of sulphadimidine for 1, 2, 3, and 4 weeks respectively. All of the mice in the first two groups, 3 of those in the third and only 1 of the fourth group relapsed after treatment was stopped. A comparable experiment with pyrimethamine showed that, if treatment lasted 1 or 2 weeks, all the animals relapsed ; whereas, if treated for 4 weeks, very few relapsed. As a result of these preliminary trials, it was decided that in further experiments the drugs would be given for 4 weeks. Mice which relapsed did so most frequently in the second and third weeks after cessation of treatment.

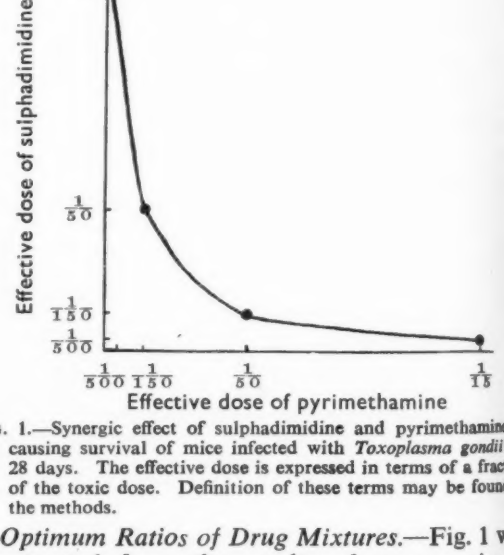

FIG. 1.—Synergic effect of sulphadimidine and pyrimethamine in causing survival of mice infected with *Toxoplasma gondii* for 28 days. The effective dose is expressed in terms of a fraction of the toxic dose. Definition of these terms may be found in the methods.

Optimum Ratios of Drug Mixtures.—Fig. 1 was constructed from the results of an experiment using various combinations of doses of sulphadimidine and pyrimethamine. It shows that the combination which produced an effect on toxoplasmosis with the least summation of toxicity was

1/100 TD of each compound. Of the 24 groups of mice, there were 4 in which all animals survived the 4-week period of treatment, but nearly all relapsed or were proved to be carriers. In a similar experiment using combinations of dapsone and pyrimethamine, 1/16 TD of each compound was found to be the optimum ratio for an effect comparable with that of the former experiment (Fig. 2). These experiments show that there is

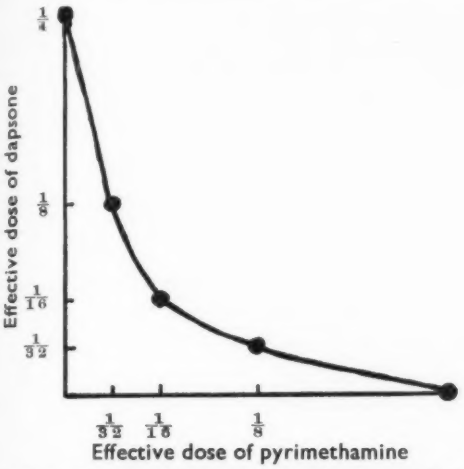

FIG. 2.—Synergic effect of dapsone and pyrimethamine in causing survival of mice infected with *Toxoplasma gondii* for 28 days. Doses, see Fig. 1.

synergy between pyrimethamine and either sulphadimidine or dapsone and that the optimum ratios of the drugs are equal fractions of their toxic doses. A third experiment showed that there was no synergy, but an additive effect between dapsone and sulphadimidine. From these preliminary results it was decided that in further experiments combinations of drugs would be used only in equal fractions of their toxic doses.

Effect of Drugs When Given Singly.—The results of giving the drugs alone are shown in Table I. Of 18 untreated control mice used in these experiments, all died within 6 to 9 days (average 7½).

Effect of Combinations of Drugs.—The results of giving the drugs in combinations are also shown in Table I. These experiments were performed at the same time and using the same controls as those in which the drugs were given singly.

At the end of the treatment period all the surviving mice were weighed. The gains in weight were more than those of normal uninoculated mice in all groups except for those receiving (*a*) 1/2 TD of each of sulphadimidine and pyrimethamine, (*b*) 1/2 TD of each of dapsone and pyrimethamine (these two groups gained less than normally), and (*c*) 1/4 TD or more of either sulphadimidine or pyrimethamine, (*d*) 1/4 TD of each of dapsone and pyrimethamine, (*e*) 1/2 TD each of dapsone and sulphadimidine (these three groups gained normally). There did not appear to be any synergy of toxicity.

TABLE I

RESULTS OF TREATMENT OF TOXOPLASMOSIS IN MICE WITH DRUGS GIVEN SINGLY AND IN PAIRS

See Methods for the definition of Toxic Dose (TD). The column headed Response gives the number of mice out of the groups of 6 which were completely cured (A), which survived 4 weeks (B) and which survived 1 week longer than the controls (C). The numerals in brackets give the number of survivors in each group which were subsequently found to be carriers.

Drug	Dose (Fraction of TD)	Response		
		A	B	C
Sulphadimidine ..	1/2	5	6	6
	1/4	2	6	6
	1/8	4	6	6
	1/16	1	6	6
	1/32	4	6	6
	1/64	0 (2)	2	5
	1/128	0	0	0
Pyrimethamine ..	1/2	6	6	6
	1/4	0 (2)	6	6
	1/8	0	0	3
	1/16	0	0	0
Dapsone	1/2	0	6	6
	1/4	1 (3)	6	6
	1/8	0 (1)	1	4
	1/16	0	0	0
Sulphadimidine + pyrimethamine ..	1/2	6	6	6
	1/4	5	6	6
	1/8	6	6	6
	1/16	6	6	6
	1/32	6	6	6
	1/64	3	6	6
	1/128	3 (1)	4	5
	1/256	0	0	0
Dapsone + pyrimethamine ..	1/2	4	6	6
	1/4	4	6	6
	1/8	6	6	6
	1/16	2 (1)	6	6
	1/32	2 (2)	5	6
Sulphadimidine + dapsone ..	1/2	3	6	6
	1/4	4	6	6
	1/8	3	6	6
	1/16	4	6	6
	1/32	1	6	6
	1/64	0 (5)	5	5
	1/128	0	0	2
	1/256	0	0	0

TABLE II

RESULTS OF DELAYED TREATMENT OF TOXOPLASMOSIS IN MICE WITH 1/4 TOXIC DOSE OF EACH OF SULPHADIMIDINE AND PYRIMETHAMINE

See Methods for definition of Toxic Dose. Response recorded as in Table I.

Interval between Inoculation and Start of Treatment (Days)	Response		
	A	B	C
0	6	6	6
1	6	6	6
2	2	4	6
3	3 (1)	4	5
4	0	0	1

Delayed Treatment with Sulphadimidine and Pyrimethamine Combined.—The results of treatment with 1/4 TD of each of sulphadimidine and pyrimethamine commencing at different times after inoculation are shown in Table II. Six untreated control mice died within 6 to 9 days (average 6½). Most of those mice whose treatment was delayed until 96 hr. were already sick and ate little of the medicated diet.

Autopsy Findings.—In mice which died within 6 to 9 days, organisms were usually most abundant in the lungs; in those which died within 10 to 12 days, in the liver; and those which died within 13 to 14 days, in the spleen. In mice which died still later, toxoplasms were found most easily in the brain. In mice which relapsed after treatment had ceased, there was a similar distribution of parasites in their organs according to the time of their death.

Blood Concentrations of Drugs.—After 3 days of treatment with diet containing 1/2 TD of drug, the concentrations of sulphadimidine were 214, 166, 208, 220, 192, and 164 μg./ml. of blood (average, 194 μg./ml.) and those of dapsone were 4.1, 6.4, 3.4, 3.2, 4.0, and 4.6 μg./ml. of blood (average, 4.3 μg./ml.). Attempts were made to assay pyrimethamine in the blood by the technique of Schmidt, Hughes and Schmidt (1953), but the method was not sufficiently sensitive.

Discussion

From the results it would appear that sulphadimidine alone is more active than either pyrimethamine alone or dapsone alone against the acute stages of toxoplasmosis in mice.

For the prevention of death during the acute stage of the infection there does not appear to be any advantage in combining pyrimethamine either with dapsone or sulphadimidine, but for the prevention of chronic infection a combination of sulphadimidine with pyrimethamine is much superior to the other combinations tried, or to any drug alone. This combination is active if withheld until infection is well established.

There is definite synergy between sulphadimidine and pyrimethamine and this is most marked if the criterion of effectiveness is a complete elimination of infection. There is also synergy between dapsone and pyrimethamine, less marked than with the former pair of drugs when considering complete cures, but more marked when considering treatment of the acute stages.

Nearly all the carriers (Tables I and II) were found in groups of mice which had been given only just sufficient drug to prevent death during the treatment period. It is possible that in these animals the infection was only partially controlled and that sufficient multiplication of organisms took place to stimulate active immunity which prevented relapse when treatment ceased.

Mice which had received larger doses of drugs and which subsequently relapsed did not become carriers, but died. This might be because during the treatment period the toxoplasms were present either in numbers too small to act as an antigenic stimulus, or in a form such as a terminal colony or a cyst which may be incapable of acting as an antigen. On stopping treatment proliferation would occur. Synergy between pyrimethamine and either sulphadimidine or dapsone may be connected with the fact that, whilst sulphadimidine and dapsone are competitors of *p*-aminobenzoic acid, pyrimethamine is a competitor of folic acid (Hitchings, 1952). If toxoplasms normally synthesize their own folic acid, one would expect drugs of the sulphonamide type to act against them. These drugs may not, however, be effective either in preventing the formation of cysts or in eliminating organisms already in cysts. In this phase there is no active growth and the organisms presumably contain sufficient folic acid for their reduced metabolism. Consequently, in an animal given dapsone or sulphadimidine alone, not all of the parasites would be eradicated and, if immunity had not been acquired in the meantime, relapses would occur on cessation of treatment. However, since it is possible that pyrimethamine can interfere with the metabolic effects of folic acid whether synthesized by the parasite or absorbed from the tissue fluids of the host, the combination of dapsone or sulphadimidine with pyrimethamine could lead to the complete eradication of the parasite. The reason why such a large dose of pyrimethamine is required when given alone may be that toxoplasma synthesize so much folic acid that an effective concentration of the drug is one which is near the level toxic to the host. If the rate of synthesis of folic acid by the parasite is reduced by a sulphonamide drug, less pyrimethamine is required.

We suggest that sulphadimidine interferes with the synthesis of folic acid in toxoplasma and that it may be especially effective against the proliferative forms found in acute infections. It may not, however, be able to prevent the development of cysts or affect the relatively inactive parasites in terminal colonies or cysts. It is suggested that the latter may rely on the slow metabolism of their stores of folic acid and that pyrimethamine may

interfere with such metabolism and thus prevent subsequent relapse or development of the carrier state. Pyrimethamine may even prevent the development of terminal colonies and cysts.

We wish to thank Professor C. P. Beattie for his unstinted advice and encouragement and Miss G. Calton for her valuable assistance with the technical work. The work was assisted by a grant from the Medical Research Council.

REFERENCES

Beverley, J. K. A., and Fry, B. A. (1956). *J. gen. Microbiol.*, **14**, ii.

Bratton, A. C., and Marshall, E. K., Jnr. (1939). *J. biol. Chem.*, **128**, 537.

Cross, J. B. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 548.

Eyles, D. E., and Coleman, N. (1952). *Publ. Hlth Rep., Wash.*, **67**, 249.

—— —— (1953a). *Amer. J. trop. Med.*, **2**, 54.

—— —— (1953b). *Antibiot. & Chemother.*, **3**, 483.

Francis, J., and Spinks, A. (1950). *Brit. J. Pharmacol.*, **5**, 565.

Hitchings, G. H. (1952). *Trans. R. Soc. trop. Med. Hyg.*, **46**, 467.

Reed, L. J., and Muench, H. (1938). *Amer. J. Hyg.*, **27**, 493.

Sabin, A. B. (1941). *J. Amer. med. Ass.*, **116**, 801.

Schmidt, L. H., Hughes, H. B., and Schmidt, I. C. (1953). *J. Pharmacol.*, **107**, 92.

Summers, W. A. (1949). *Amer. J. trop. Med.*, **29**, 889.

Warren, J., and Sabin, A. B. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 15.

Brit. J. Pharmacol. (1957), **12**, 194.

OXYTOCIC ACTIVITY OF BASIC (AMINOMETHYL) DERIVATIVES OF PHENOLS AND RELATED COMPOUNDS

BY

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A series of piperidinomethyl and related derivatives of naphthols, substituted phenols and indoles has been tested for oxytocic activity, using *in vitro* and *in vivo* methods of assay. Some of the compounds possessed very high activity, exceeding that of ergometrine. Activity was not associated with any structural resemblance to the ergot alkaloids.

Highest activity occurred with 2-piperidinomethyl derivatives of phenols, among which maximum potency was conferred by substitution, at both the 4- and 5- positions, by methyl or ethyl or by linkage of these positions to form an indane derivative. In all series, piperidinomethyl derivatives were more active than those formed with other bases and methylation in the position α- to the nitrogen atom augmented the activity of both piperidine and morpholine derivatives. Among 2′-methyl piperidinomethyl phenols, the (−) form was more active than the (+). Acylation or alkylation of the phenolic hydroxyl group did not affect activity.

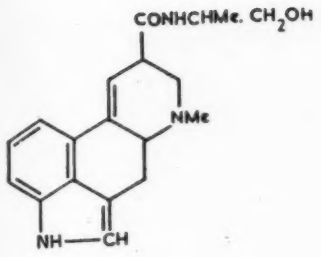
The oxytocic activity was specific, the compounds being less effective upon other forms of smooth muscle. Effects upon blood pressure and respiration of a central nature were observed.

The elaboration of synthetic drugs based on a simplification of the molecular structure of a natural prototype has been markedly successful in the fields of analgesics and curare-like compounds. Similar studies have been directed in recent years to the search for synthetic oxytocics. Much of this work has been devoted to compounds representing fragments of the structure of ergometrine (I).

Thus Baltzly and his co-workers (Baltzly, Dvorkovitz and Phillips, 1949) reported 5 to 10% of ergometrine activity in II.

Bovet and his co-workers based their studies on the tetrahydro-2-naphthylamine fragment of ergometrine. Compounds III and IV (Marini-Bettòlo, Chiavarelli and Bovet, 1950; Marini-Bettòlo, Chiavarelli and Bovet-Nitti, 1951) were found active (Bovet-Nitti, 1952). The structures could be considerably simplified to N:N′-tetraethylglycineamide which was highly active (Bovet-Nitti, 1952). Considerable activity was also found among substituted N-phenylglycineamides (Bovet-Nitti and Bovet, 1954). Gearien and Liska (1954) found activity in V and VI, among naphthalenic structures related to ergometrine, and, to a lesser extent, in alkylated glycineamides (Rosen, Blumenthal, Townsend, Tislow and Seifter, 1956). Another active series of tetrahydro-2-naphthylamine derivatives has been reported by Kraushaar (1954). The substituted 3-phenylpiperidine derivative (VII) has also been found active (Plieninger, 1953).

Akkerman and Veldstra (1954) and van Proosdij-Hartzema and de Jongh (1954) studied compounds of the "open-ergometrine" type (VIII), but found them far less active than the more readily prepared isomeric piperidinomethylindoles (IX) which, however, are less closely related to ergometrine (Akkerman, de Jongh and Veldstra, 1951; de Jongh and van Proosdij-Hartzema, 1952). The suggestion that oxytocic activity may not require a true partial structure of ergometrine receives further support from examples such as the simple oxytocics of Bovet (see above); in addition, significant activity has been reported for the piperidinomethylindene



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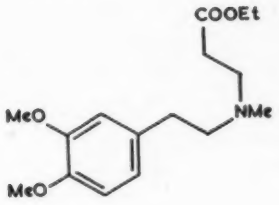

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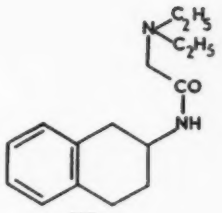

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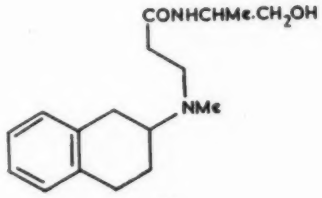

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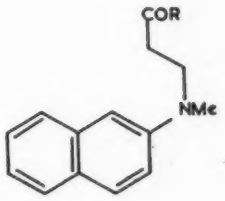
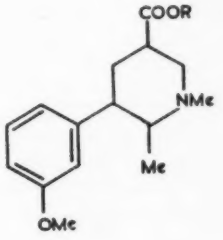


V, R=OEt
VI, R=NEt$_2$



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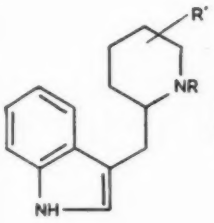

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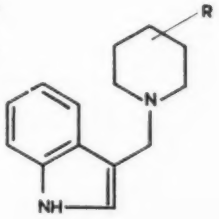

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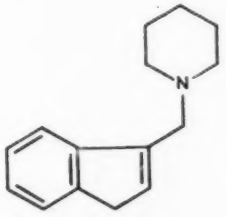

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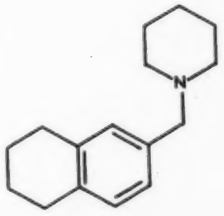

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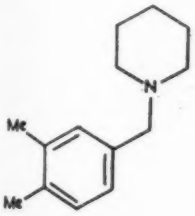

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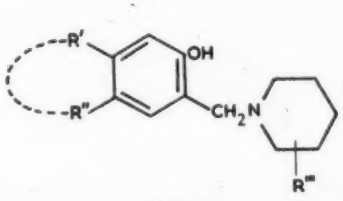

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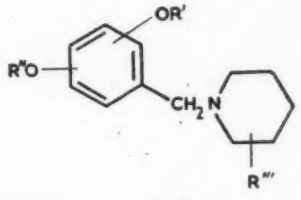


XIV

(X) by Hoffmann and Schellenberg (1944) and for compounds such as XI and XII by Schindler and Voegtli (1949).

It therefore appeared worth while seeking oxytocic activity outside the partial structures of ergometrine. The compounds studied in the present investigation were mainly substituted aminomethylphenols (XIII) prepared by the Mannich reaction of various secondary amines, formaldehyde and a series of phenolic substances in place of indole as used by Akkerman *et al.* (1951).

While this work was in progress information became available of the oxytocic properties of substituted N-benzylpiperidines disclosed in U.S. Patent 2589205 which included compounds closely related to or identical with a few discussed below. Another U.S. Patent, 2633468, claimed

active piperidinomethylcoumarans. More recently the activity of hydroxy- and methoxy-substituted N-benzyl piperidines (XIV) has been reported by Hejno and Arnold (1953) and Arnold and Hejno (1955).

METHODS

Isolated Guinea-pig Uterus.—Each substance was assayed by direct comparison with an oxytocic standard, usually ergometrine maleate, using a method based on that of Dale and Laidlaw for assaying pituitary extract as recommended by the *British Pharmacopoeia* (1948) and Burn (1937). One horn of the uterus from a virgin guinea-pig of 160–300 g. weight was suspended in modified Ringer solution at 36° C. containing NaCl 0.9%, KCl 0.042%, $CaCl_2$ 0.024%, $MgCl_2$ 0.044%, $NaHCO_3$ 0.05%, and dextrose 0.05%. The large quantity of magnesium chloride was adopted to reduce spontaneous activity and to sensitize the uterus to stimulation (Hsu, 1948).

Substances were assayed by a bracketing procedure, four doses constituting one comparison, comprising one dose level of test substance (T) and one dose level of standard (S), each repeated twice in a sequence STTS or TSST. Dose levels were chosen to produce contractions about 70% of maximal. Each dose was allowed to act for 3 min., when the fluid was changed and the organ rested for 6 min. before the next dose. Groups of doses were repeated, being adjusted until comparisons were obtained showing the smallest dose T producing responses greater than those due to S and also the largest dose of T which was less effective than a dose of S (Fig. 1). This gave the range within which the relative activity lay. Each assay was repeated until the range in at least two tests was narrower than ±33% of the mean value.

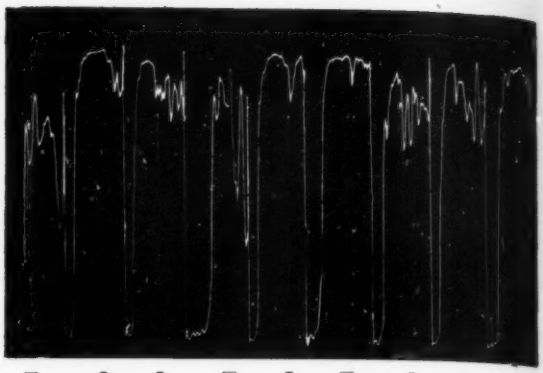


FIG. 1.—Isolated guinea-pig uterus. Bath volume 30 ml. At T_1, Ro 3–0601 0.3 mg.; at T_2, Ro 3–0601 0.5 mg.; at S, ergometrine maleate 0.01 mg. In the first set of four doses, $S > T_1$; in the second set $T_2 > S$. From this, Ro 3–0601 showed between 2 and 3% the activity of ergometrine, by weight.

Guinea-pig Uterus in situ.—Virgin guinea-pigs, weighing 200 to 600 g., were anaesthetized with urethane, 2.4 g./kg. subcutaneously, and uterine movements recorded by a modification of a method described by Bell and Robson (1936). One horn of the uterus was exposed through an abdominal incision and a silver wire hook attached to a thread was passed through the horn at its mid-point or round it by piercing the ligament below it. The thread was passed through an inverted funnel which was clamped in position over the uterus, excluding other viscera but with care not to bear on the uterus or restrict its

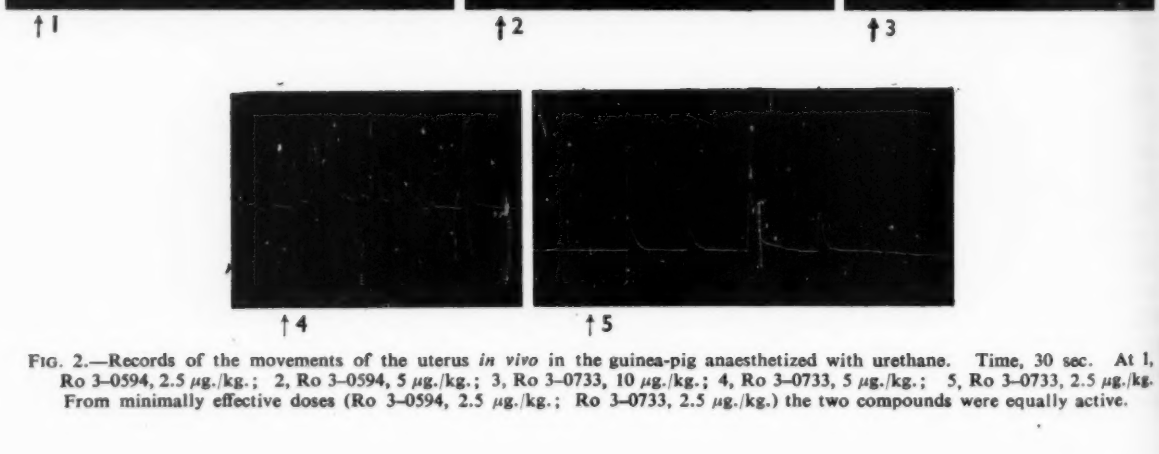


FIG. 2.—Records of the movements of the uterus *in vivo* in the guinea-pig anaesthetized with urethane. Time, 30 sec. At 1, Ro 3–0594, 2.5 μg./kg.; 2, Ro 3–0594, 5 μg./kg.; 3, Ro 3–0733, 10 μg./kg.; 4, Ro 3–0733, 5 μg./kg.; 5, Ro 3–0733, 2.5 μg./kg. From minimally effective doses (Ro 3–0594, 2.5 μg./kg.; Ro 3–0733, 2.5 μg./kg.) the two compounds were equally active.

circulation. The abdominal wall was closed round the funnel and the thread attached to a frontal lever weighted to lift the horn so that subsequent contraction exerted tension on the thread. The ends of the horn were not attached to the funnel, as in the method of Bell and Robson, so as to avoid interference with the uterus and to minimize spontaneous activity and insensitivity to stimulation. Drugs were injected into the jugular vein. Activity was measured in comparison with a standard by determining the minimally effective dose for each (Fig. 2).

Cat Uterus in situ.—Cats were anaesthetized with chloralose, 80 mg./kg. intravenously under light ether. Movements of one horn of the uterus were recorded by the method described for guinea-pigs. Recordings of blood pressure were made from the carotid or femoral artery using a mercury manometer or a membrane manometer, of heart rate using the method of Dawes (1951), and respiration by the method of Paton (1949). Drugs were injected into the saphenous vein.

Perfused Rabbit Ear and Rat Hindquarters.—Rabbits were stunned and bled. After cannulating the central artery, the ear was removed. Rats were stunned and bled. The aorta was cannulated near the iliac bifurcation and the hindquarters were separated from the remainder of the animal. Both preparations were perfused from a Mariotte bottle with modified Locke solution containing NaCl, 0.9% ; KCl, 0.042% ; $CaCl_2$, 0.024% ; $NaHCO_3$, 0.05% ; dextrose, 0.05%. Drugs were injected in small volumes through a rubber cap on a side arm near the tip of the cannula.

Bronchoconstrictor Effect. — Guinea-pigs were pithed under ether and the resistance of the bronchial tree to inflation by artificial respiration was registered by a piston recorder using the principle of Konzett and Rössler (1940). Drugs were injected into the jugular vein.

RESULTS

Oxytocic Activity in vitro.—The activity of a number of piperidinomethyl derivatives of phenolic compounds is shown in Table I. Values are expressed on a molar basis, relative to ergometrine as unity. Among the first six compounds tabulated the greatest activity was found in the 2-piperidinomethyl-4 : 5-dimethylphenol (**5**), which was twice as active as ergometrine. Analogous compounds from isomeric xylenols (**7** to **10**; **29, 30**), were all of lower activity than **5**. Additional methyl substitution in the phenolic ring generally reduced activity (**11, 12**). Among the 2:4:5-trisubstituted phenols homologous with 5 activity was unchanged on enlarging the 5-substituent to ethyl (**18**) although increase of the 4-substituent to ethyl augmented activity, both with 5-methyl (**13**) and 5-ethyl (**19**). Further enlargement of the 4-substituent to propyl or *iso*propyl (**14, 15**) diminished activity. The negligible activity of the 4-propyl compound (**16**) which is isomeric with **13** and **18** suggests that substitution of both 4- and 5-positions is necessary.

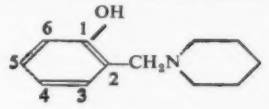
The indane derivative **21**, which also showed high activity, may be considered as containing these substituents linked to form the additional ring. No compound containing an additional 6-membered ring showed high activity.

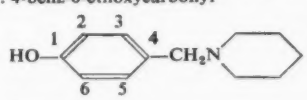
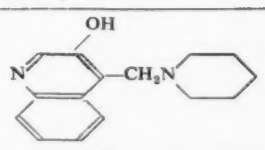
Table II shows the activities of compounds in which various basic groups are linked to 3:4-xylenol, 2-naphthol and indole. In the first series, the piperidine ring (3-0575) confers greater activity than does the pyrrolidine (2-6428), hexamethyleneimine ring (3-0685), or any other secondary amine. The superiority of piperidine to morpholine is seen in all three series (3-0575 and 3-0710). Linkage of the basic group to the

TABLE I

OXYTOCIC ACTIVITIES OF PIPERIDINOMETHYL DERIVATIVES OF PHENOLIC COMPOUNDS

Molar activities on isolated guinea-pig uterus (ergometrine=1).



No.	Substituents	Activity
Ro		
1. 3–0737		0·02
2. 3–0677	5-methyl	0·03
3. 3–0676	4-methyl	0·03
4. 3–0696	4-chloro	0·03
5. 3–0575	4: 5-dimethyl	2·0
6. 3–0698	4-chloro-5-methy	0·5
7. 3–0590	3: 5-dimethyl	0·8
8. 3–0624	5: 6-dimethyl	0·2
9. 3–0635	4: 6-dimethyl	0·15
10. 3–0682	3: 4-dimethyl	0·6
11. 3–0675	3: 4: 6-trimethyl	0·3
12. 3–0711	3: 4: 5-trimethyl	0·65
13. 3–0707	4-ethyl-5-methyl	5·5
14. 3–0734	4-propyl-5-methyl	0·6
15. 3–0777	4-*iso*propyl-5-methyl	0·3
16. 3–0748	4-propyl	<0·01
17. 3–0761	4-*cyclo*hexyl	<0·01
18. 3–0739	4-methyl-5-ethyl	2·0
19. 3–0733	4: 5-diethyl	3·5
20. 3–0724	4: 5-dimethoxy	0·04
21. 3–0636	4: 5-trimethylene	1·25
22. 3–0542	4: 5-tetrahydrobenz	0·15
23. 3–0674	4: 5-benz	0·15
24. 3–0514	3: 4-benz	0·3
25. 3–0533	5: 6-benz	0·04
26. 3–0548	3: 4-(3′: 2′-pyrid)	0·05
27. 3–0601	3: 4-benz-5-piperidino-methyl-6-hydroxy	0·015
28. 3–0615	3: 4-benz-6-ethoxycarbonyl	<0·1
		
29. 3–0625	2: 5-dimethyl	<0·05
30. 3–0634	2: 6-dimethyl	0·02
31. 3–0524		Nil

nucleus by a single methylene carbon resulted in greater activity than when the linkage was extended (3-0742, 3-0744) or omitted (3-0820).

The effect on activity of substitution in the piperidine ring is shown in Table III for derivatives of 3:4-xylenol, 5:6:7:8-tetrahydro-2-naphthol, 2-naphthol and indole. Within the group of piperidine substituents tabulated, the 2-methyl group alone increased activity (3-0594, 3-0609, 3-0530), whereas other substitution reduced activity or had little effect on it. The advantage conferred by a 2-methyl group extended also to

TABLE II

OXYTOCIC ACTIVITIES OF BASIC DERIVATIVES OF PHENOLS, NAPHTHOLS, AND INDOLES

Molar activities on isolated guinea-pig uterus (ergometrine = 1·0).

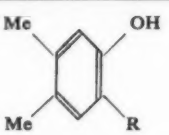
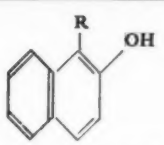
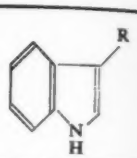
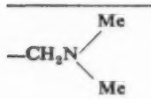
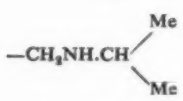
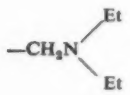
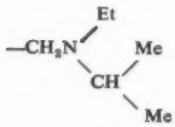
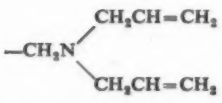
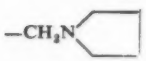
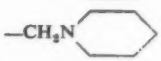
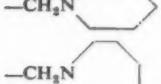
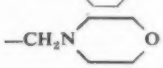
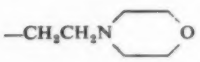
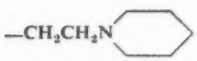
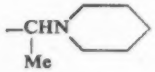
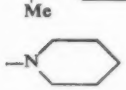
R						
	No.	Activity	No.	Activity	No.	Activity
	Ro		Ro		Ro	
	3-0859	0·03	3-0515	0·02		
	3-0701	0·12				
	3-0702	0·25				
	3-0699	0·25				
	3-0760	0·2				
	2-6428	0·5				
	3-0575	2·0	3-0514	0·3	3-0516	0·04
	3-0685	0·6				
	3-0710	0·1	3-0522	<0·005	3-0532	0·005
	3-0742	0·015				
	3-0744	0·3			3-0754	0·01
			3-0663	<0·02		
	3-0820	<0·05				

TABLE III

OXYTOCIC ACTIVITIES OF SUBSTITUTED PIPERIDINOMETHYL DERIVATIVES OF PHENOLS, NAPHTHOLS, AND INDOLES

Molar activities on isolated guinea-pig uterus (ergometrine = 1·0).

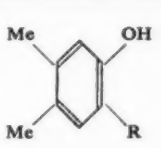
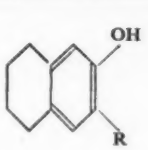
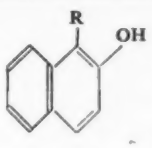
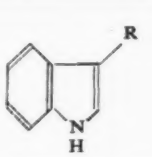
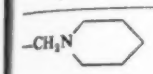
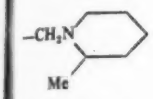
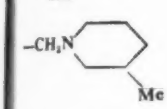
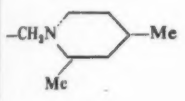
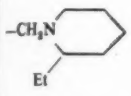
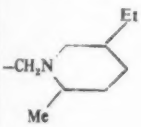
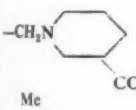
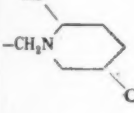
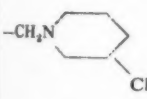
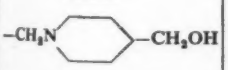
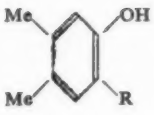
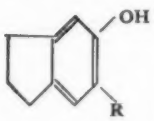
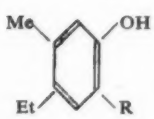
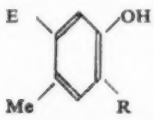
R								
	No.	Activity	No.	Activity	No.	Activity	No.	Activity
	Ro 3-0575	2·0	Ro 3-0542	0·15	Ro 3-0514	0·3	Ro 3-0516	0·04
	3-0594	4·0	3-0609	0·33	3-0540	0·12	3-0530	0·1
	3-0632	0·8			3-0612	0·1		
	3-0627	0·66			3-0541	0·1	3-0544	0·1
	3-0614	0·66			3-0545	0·15		
	3-0712	0·66						
	3-0626	0·12			3-0536	0·03	3-0537	< 0·001
	3-0633	0·6						
	3-0611	< 0·01	3-0610	0·2	3-0570	0·4	3-0535	Nil
	3-0722	0·2						
					3-0599	0·05		
	3-0628	0·04			3-0600	0·15		
	3-0631	0·01						

TABLE IV

EFFECT UPON OXYTOCIC ACTIVITY OF SUBSTITUTING PIPERIDINE OR MORPHOLINE GROUP BY METHYL IN α-POSITION

Molar activities (ergometrine=1·0). The *in vivo* results with cats and those marked with an asterisk were obtained by comparison with Ro 3–0594 and are expressed as molar activities relative to Ro 3–0594=4·0 (see text).

	R=—CH_2N⟨piperidine⟩				R=—CH_2N⟨2-Me-piperidine⟩				R=—CH_2N⟨morpholine⟩				R=—CH_2N⟨2-Me-morpholine⟩			
	No.	Guinea-pig		Cat	No.	Guinea-pig		Cat	No.	Guinea-pig		Cat	No.	Guinea-pig		Cat
		In vitro	*In vivo*	*In vivo*		*In vitro*	*In vivo*	*In vivo*		*In vitro*	*In vivo*	*In vivo*		*In vitro*	*In vivo*	*In vivo*
	Ro				Ro				Ro				Ro			
	3–0575	2·0	1·5		3–0594	4·0	4·0	4·0	3–0710	0·1	0·3*	1·0	3–0738	0·53	4·5*	3·3
	3–0636	1·25	0·6	0·6	3–0670	3·0	6·0*	1·2	3–0745	0·05	0·25*		3–0747	0·33	2·3	2·5
	3–0707	5·5	12·5	6·5	3–0713	13·0	3·3*	6·6								
	3–0739	2·0	3·2*	0·6	3–0752	3·0	4·0	1·5								

morpholine derivatives as seen in Table IV (3-0738), which also shows this effect in the 5-hydroxyindane series (3-0670, 3-0747).

Oxytocic Activity in vivo.—In view of the different criteria adopted in evaluating activity *in vitro* and *in vivo* in the guinea-pig, there was no reason to suppose that results from the two methods would correspond. Substances tested *in vitro* were compared at dose levels causing nearly maximal responses, while in the *in vivo* method minimally effective doses were sought. Nevertheless, Tables IV and V show that, in general, fairly close parallelism was observed. Similar agreement was also observed between *in vitro* and *in vivo* results from cat and rabbit uteri.

However, the morpholine and methylmorpholine compounds (Table IV) showed activities in both guinea-pig and cat uterus *in situ* which were consistently greater, by a factor of between 3 and 10, than those in the isolated uterus. We consider that these differences between *in vitro* and *in vivo* results indicate a reduction in relative potency at responses approaching maximal, as used in the *in vitro* test, as these compounds show less steeply inclined dose-response curves than those of the other oxytocics studied.

Owing to an inconsistency of response towards ergometrine observed in the uteri of all species tested *in vivo*, it was necessary to use another compound as a subsidiary standard and it was found convenient to test many substances against

TABLE V

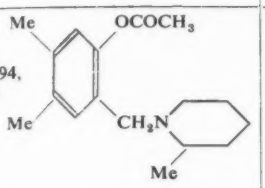
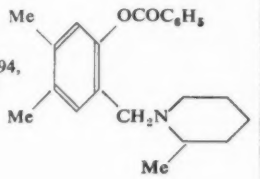
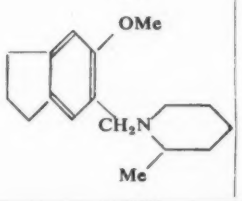
IN VITRO AND *IN VIVO* ACTIVITIES OF OXYTOCIC COMPOUNDS

Molar activities (ergometrine=1.0). The *in vivo* results marked with an asterisk were obtained by comparison with Ro 3–0594 and are expressed as molar activities relative to Ro 3–0594=4.0 (see text).

No. Ro	Guinea-pig		No. Ro	Guinea-pig	
	In vitro (Tables I–III)	*In vivo*		*In vitro* (Tables I–III)	*In vivo*
3–0514	0·3	0·6	3–0570	0·4	0·1
3–0515	0·02	0·01	3–0590	0·8	0·8
3–0516	0·04	0·07	3–0614	0·66	1·0
3–0522	<0·005	0·05	3–0632	0·8	0·8
3–0530	0·1	0·15	3–0702	0·25	0·6
3–0537	<0·001	<0·02	3–0712	0·66	0·66*
3–0540	0·12	0·6	3–0733	3·5	3·5*
3–0545	0·15	0·6	3–0734	0·6	
			3–0760	0·2	

TABLE VI

OXYTOCIC ACTIVITIES OF BASIC DERIVATIVES OF ACYLATED AND ALKYLATED PHENOLS

No.	Oxytocic Activity (Ergometrine=1·0) Guinea-pig Uterus	
	In vitro	*In vivo*
Ro 3-0862 (cf. Ro 3-0594, Table IV) 	4·8	4·0
Ro 3-0867 (cf. Ro 3-0594, Table IV) 	3.8	2.6
Ro 3-0885 (cf. Ro 3-0670, Table IV) 	1·0	

Ro 3-0594 which had itself been standardized against ergometrine.

Effect of Modifying the Phenolic Group.—Two O-acyl derivatives, Ro 3-0862 and Ro 3-0867, and one O-methyl ether, Ro 3-0885, exerted similar oxytocic activity, *in vitro* and *in vivo*, to the parent phenols (Table VI).

They also produced effects upon the blood pressure and respiration of the chloralosed cat similar to those of the parent phenols.

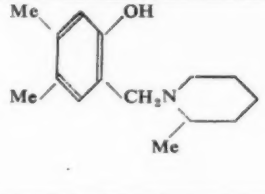
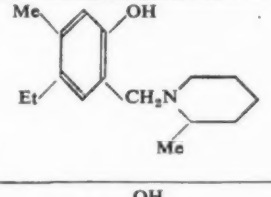
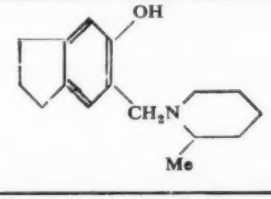
Effect of Optical Resolution.—The (−) and (+) forms of α-pipecoline were used to prepare optical isomers of three derivatives, 2-(N-2′-methylpiperidinomethyl)-4:5-dimethylphenol, 2-(N-2′-methylpiperidinomethyl)-4-ethyl-5-methylphenol and 6-(2′-methylpiperidinomethyl)-5-hydroxyindane, the racemic forms of which appear in Table IV as Ro 3-0594, Ro 3-0713 and Ro 3-0670 respectively. Table VII shows the relative activities of the (−), (+) and racemic forms of each and it may be seen that greater activity was found for the (−) forms, although the (+) forms were also considerably active.

General Pharmacology.—In contrast to our experience with ergometrine, all uteri used responded consistently to these synthetic oxytocics.

TABLE VII

OXYTOCIC ACTIVITIES OF OPTICAL ISOMERS OF α-PIPECOLINOMETHYL DERIVATIVES OF SUBSTITUTED PHENOLS

The *in vivo* results were obtained by comparison with Ro 3–0594 and are expressed as molar activities relative to Ro 3–0594 = 4·0.

No. Ro		Oxytocic Activity (Ergometrine=1·0) Guinea-pig Uterus *In vitro*	Oxytocic Activity (Ergometrine=1·0) Guinea-pig Uterus *In vivo*	Oxytocic Activity (Ergometrine=1·0) Cat Uterus *In vivo*	LD50 i.v. in Mice (mg./kg.) (P=0·05)
3-0694	 (−)	6·0	11·0	12·0	17·5 (16·2–19)
3-0679	(+)	0·8	1·6	1·6	81·0 (74·5–88·5)
3-0594	(±)	4·0	4·0	4·0	57·0 (54·5–59·5)
3-0765	 (−)	14·5	4·3	7·25	25 (21–31)
3-0771	(+)	2·3	0·85	1·1	35 (32–38)
3-0713	(±)	13·0	3·3	6·6	
3-0857	 (−)	5·2	11·0		33 (28–39)
3-0858	(+)	2·1	2·0		80
3-0670	(±)	3·0	6·0		41 (38–46)

No difference in sensitivity has been found with differences in age of animal or condition of the uterus.

The stimulant action of this class of compounds affects the uterus more than other forms of smooth muscle. Whereas isolated guinea-pig uterus contracted in response to dilutions of 1 in 10^7 of the more active compounds, isolated guinea-pig ileum and seminal vesicle required dilutions of the order of 1 in 10^5 for minimal response. Isolated rabbit ileum relaxed in response to a dilution of 1 in 10^6 (Fig. 3). The compounds were vasoconstrictors and weak antagonists of adrenaline on the perfused rabbit ear and rat hindquarters preparations (Fig. 4). They were also bronchoconstrictors in the pithed guinea-pig preparation (Fig. 5), a property shared by ergometrine.

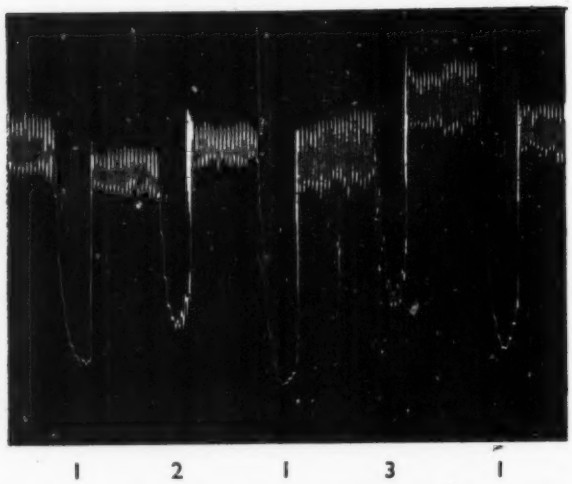


FIG. 3.—Isolated rabbit ileum. Bath volume, 30 ml. At 1, adrenaline 4 μg. 2, Ro 3-0575 30 μg. 3, Ro 3-0738 30 μg.

Lethal intravenous doses of these compounds in the mouse and rabbit caused death by respiratory arrest and convulsions ; a noticeable effect of near-lethal doses was profuse salivation. Values for LD50 by the intravenous route are given for some of the compounds in Table VIII. These values may be compared with the published LD50 for ergometrine as 145 mg./kg. (Rothlin, 1935) and 114.0 mg./kg. (Akkerman *et al.*, 1951).

On injecting the chloralosed cat intravenously with oxytocic doses of the Mannich compounds, the principal effects seen were brief respiratory arrest and fluctuation of blood pressure (Fig 6) There was also a stimulatory effect on respiration ; low doses increased rate and reduced volume, while higher doses caused rapid, shallow breathing following the initial, brief arrest.

In general, low doses of these compounds given to a chloralosed cat caused a small rise in the blood pressure which was of shorter duration than the oxytocic effect. Higher doses often produced

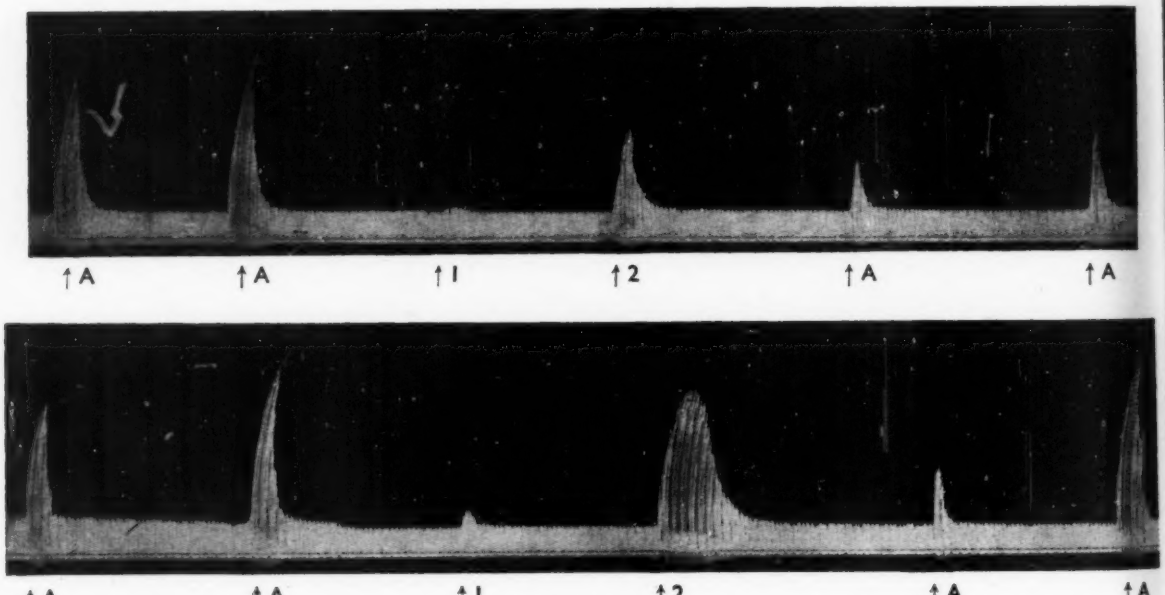


FIG. 4.—Records of outflows from the perfused isolated rabbit ear (upper trace) and the perfused rat hind-quarters (lower trace). An increase in amplitude denotes vasoconstriction. Time, 30 sec. At A, adrenaline 0.01 μg.; at 1, Ro 3–0575 1.0 mg.; at 2, Ro 3–0575 2.0 mg. The response to adrenaline was reduced after Ro 3–0575.

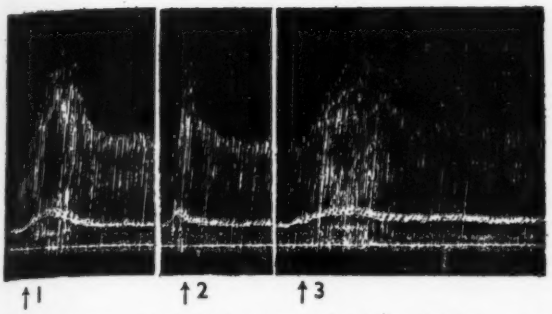


FIG. 5.—Record of bronchial resistance in the artificially respired pithed guinea-pig. A rise in bronchial tone is denoted by an increase in amplitude. Time, 30 sec. At 1, Ro 3–0575 5.0 μg./kg.; at 2, histamine 5.0 μg./kg.; at 3, Ro 3–0670 10.0 μg./kg.

TABLE VIII

LD50 VALUES FOR OXYTOCIC COMPOUNDS IN MICE (INTRAVENOUS INJECTION)

No. Ro	LD50 mg./kg. with Limits of Error in Parentheses (P=0·05)
3–0514	45·5
3–0536	15·0 (13·4–16·8)
3–0540	4·8 (3·8–6·05)
3–0545	44·0 (41·3–45·9)
3–0570	168·0 (150–189)
3–0575	37·0 (35–39·2)
3–0590	69·0 (61·5–77·5)
3–0614	60·0 (57·5–62)
3–0627	32·0 (27·1–36·9)
3–0632	37·0 (33–41·5)
3–0636	39·0 (34·5–44)
3–0707	56·0 (51–62)
3–0710	110·0 (103–117·5)
3–0738	118·0 (110–125)
3–0747	105·0 (94·5–116)

an initial, brief depression followed either by a rise in pressure (Fig. 6) or, usually with still larger doses, rhythmic fluctuations of as much as 60 mm. Hg, and at a rate of about 4/min., which were accompanied by similar fluctuations in respiratory movements (Fig. 7). Bradycardia was also observed. The effects upon blood pressure varied considerably in different animals and also with the anaesthetic used. Under barbiturate anaesthesia, the compounds caused only a moderately prolonged fall in blood pressure. It was notable that similar variability occurred in the response of cats to ergometrine, which was also pressor with chloralose and depressor with barbiturate anaesthesia.

Treatment with atropine, although it reduced the bradycardia, did not alter the brief depressor effect of these compounds in the chloralosed cat. This was abolished on cutting the vagi, which also prevented the effects upon respiration and further reduced the bradycardia (Fig. 7). The effect on the blood pressure was now pressor, and this was thought to be a centrally mediated action since it did not occur in the spinal cat; in this preparation depressor effects were observed with higher doses.

It is concluded that the effects upon blood pressure are due principally to two actions; a central pressor action, and a depressor effect which together with respiratory arrest and bradycardia

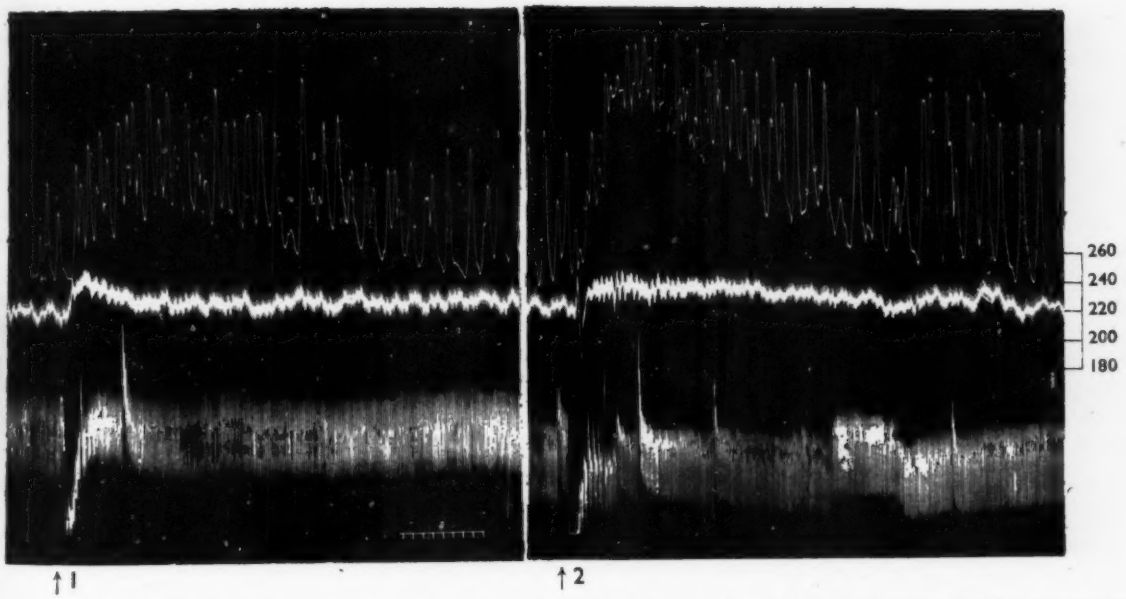


FIG. 6.—Cat anaesthetized with chloralose. The records are, from above downwards, of uterine movement, blood pressure and respiration. Time, 30 sec. At 1, Ro 3–0738, 10.0 μg./kg., and at 2, 20.0 μg./kg.

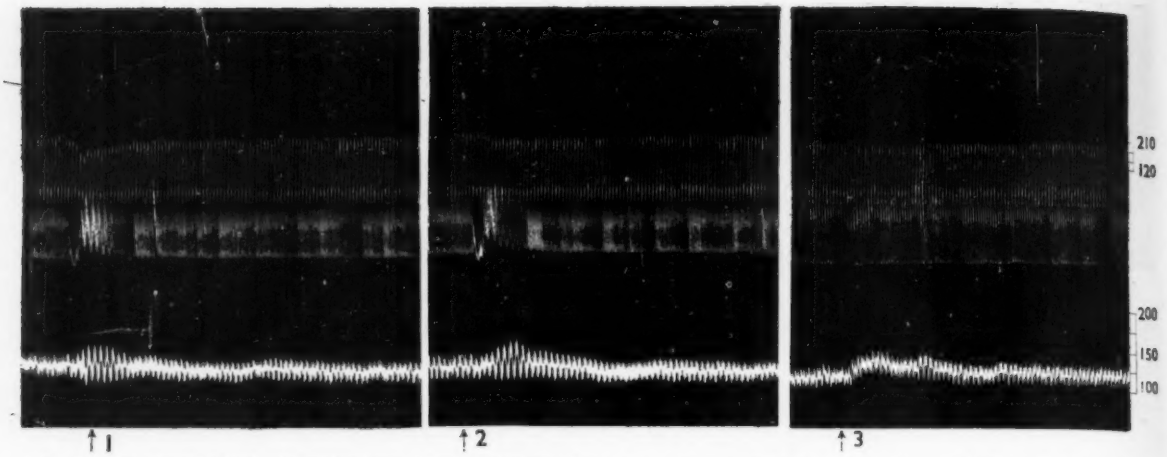


FIG. 7.—Cat anaesthetized with chloralose. The records are, from above downwards, of uterine movement, heart rate, respiration, fore-paw volume and blood pressure. Time, 30 sec. Ro 3–0707 10.0 μg./kg. at 1, 2, 3. Between 1 and 2, atropine 1.0 mg./kg., s.c. Between 2 and 3 both vagi were cut.

are mediated by the vagus. This may involve respiratory and cardiovascular reflexes resembling the Bezold reflex, similar to those excited by the veratrum alkaloids and a number of other compounds (Dawes, 1952). The resultant interaction of these effects recalls the situation found by Brown and Dale (1935) for the actions of ergometrine upon blood pressure.

DISCUSSION

Among the compounds described, many have been found to possess high oxytocic activity. The most active are several times as active as ergometrine. These basic derivatives of simple phenols have thus been shown to evoke contractions of uterine muscle more readily than any other type of synthetic compound and this is the more notable since, unlike many types studied by other workers, no structural resemblance to ergometrine can be seen in these compounds.

The simplicity of the compounds studied has enabled a series of different but closely related compounds to be examined for the effect of molecular structure upon activity. Maximum activity in this series has been shown to require, as the basic group, piperidine substituted by methyl adjacent to the nitrogen atom, and, as the nucleus, 4-methyl-5-ethyl-phenol. Divergence from this structure caused a drop in activity. The effects of changes in substituents in the basic group were similar to those described among the less active piperidinomethylindoles by Akkerman *et al.* (1951). They also found that maximum activity required a methyl substituent at either, or preferably both, of the positions adjacent to the piperidine nitrogen atom.

There is general agreement with the results of Akkerman *et al.* (1951) for the six indole derivatives Ro 3-0516, Ro 3-0530, Ro 3-0532, Ro 3-0535, Ro 3-0537 and Ro 3-0754, when our results are compared with theirs for rabbit uterus *in vivo*. However, their results for isolated guinea-pig uterus are discordant. It may be that the disagreement is due to their use of minimum effective doses as a criterion of activity *in vitro*, although our use of this criterion *in vivo* does not seem to have entailed similar discrepancies.

Uteri frequently failed to respond adequately to ergometrine *in vivo*, and the consistent effectiveness of the Mannich bases suggests that the mode of action of these compounds is not identical with that of ergometrine. Compounds based on fragments of the ergometrine molecule have not so far been found to possess more than a fraction of the oxytocic activity of the alkaloid. One molecule of the more active of the simple compounds described here, however, is as potent as 5 or more molecules of ergometrine. This, together with the absence of common structural features, supports the contention that the modes of action of these two classes of oxytocic compounds differ.

Although the compounds described here are piperidinomethyl benzene derivatives, their activity greatly exceeds that reported for the similar compounds by Schindler and Voegtli (1949) (XI and XII), and this may be attributed to the presence of an oxygen function at the *ortho*-position to the aminomethyl group. The related compounds described by Arnold and Hejno (1955) (XIII) were generally less than one-tenth as active as ergo-

TABLE IX

LIST OF COMPOUNDS

The suffixes in parentheses in column (2) refer as follows: (i) The orientation of the substituted 2-naphthols is deduced by analogy with the known 1-piperidinomethyl-2-naphthol (Caldwell and Thompson, 1939). (ii) Orientation of substituents proved by hydrogenation (Caldwell and Thompson, 1939) to 2: 4: 5-trimethylphenol; the orientation in the other substituted 3: 4-dialkylphenols is deduced by analogy. (iii) Orientation deduced by analogy with Ro 3–0542. (iv) Orientation proved by hydrogenation to 2: 3: 6-trimethylphenol. (v) Structure by analogy with Ro 3–0670. (vi) Orientation proved by hydrogenation which yielded 5-hydroxy-6-methylindane, m.p. 86° to 88° C. (benzoate, m.p. 113°–114.5° C.). Fieser and Lothrop (1936) give m.p. 83°–84° C. and 111°–112° C., respectively. The letters in column 3 indicate the method of preparation given in the text of the chemical section. The numerals in parentheses in column 4 denote references as follows: (1) Auwers and Dombrowski (1906); (2) Caldwell and Thompson (1939); (3) Pohland (1949); (4) Tseou and Yang (1939); (5) Julia (1955); (6) Burckhalter, Tendick, Jones, Holcomb and Rawlins (1946); (7) Akkerman, de Jongh, and Veldstra (1951); (8) Cohen and Heath-Brown (1954a); (9) Cohen and Heath-Brown (1954b). In column 5, the names of the salts have been abbreviated in the following manner: HCl = Hydrochloride, B.HCl; M = Acid Maleate, B. $HO_2C.CH:CH.CO_2H$; T = Acid Tartrate, B.$HO_2C[CH(OH)]_2.CO_2H$.

Ro 3– (1)	Name (2)	Method of Preparation (3)	Refs. (Known Bases) Phys. Consts. °C. (New Bases) (4)	Salt with M.P. °C. (5)
0524	3-Hydroxy-4-(piperidinomethyl)quinoline	*a*	m.p. 95	
0536	1-(4′-Ethylpiperidinomethyl)-2-naphthol (i)	*a*	m.p. 113	
0540	1-(2′-Methylpiperidinomethyl)-2-naphthol	*a, b*	m.p. 94–96	
0541	1-(4′-Methylpiperidinomethyl)-2-naphthol	*a*	m.p. 131·5–133·5	
0542	3-Piperidinomethyl-5: 6: 7: 8-tetrahydro-2-naphthol	*a, c, d*	m.p. 77–78	HCl: 197–198
0545	1-(2′: 4′-Dimethylpiperidinomethyl)-2-naphthol	*a*	m.p. 71–73·5	
0548	6-Hydroxy-5-(piperidinomethyl)quinoline	*a*		HCl: 214
0570	1-(3′-Ethoxycarbonylpiperidinomethyl)-2-naphthol	*a*		HCl: 100
0575	2-Piperidinomethyl-4: 5-dimethylphenol (ii)	*a*	(1)	
0590	2-Piperidinomethyl-3: 5-dimethylphenol	*a*	(1, 2)	M: 121–122
0594	2-(2′-Methylpiperidinomethyl)-4: 5-dimethylphenol (ii)	*a*	(3)	HCl: 190–192
				M: 134–136
0599	1-(3′-Hydroxymethylpiperidinomethyl)-2-naphthol	*a*		M: 157–158
0600	1-(4′-Ethoxycarbonylpiperidinomethyl)-2-naphthol	*a*		HCl: 99–101
				M: 162–163
0609	3-(2′-Methylpiperidinomethyl)-5: 6: 7: 8-tetrahydro-2-naphthol (iii)	*a*	b.p. 120/3 × 10^{-5} mm. n_D^{20} 1·552	T: 60–70
0610	3-(3′-Ethoxycarbonylpiperidinomethyl)-5: 6: 7: 8-tetrahydro-2-naphthol (iii)	*a*	b.p. 180/0·3 mm.	HCl: 100 T: 75–80
0611	2-(3′-Ethoxycarbonylpiperidinomethyl)-4: 5-dimethylphenol	*a*	b.p. 116/10^{-4} mm. n_D^{20} 1·525	
0612	1-(3′-Methylpiperidinomethyl)-2-naphthol	*a*		M: 157–158
0613	1-(2′-Methyl-5′-ethylpiperidinomethyl)-2-naphthol	*a*		M: 70
0614	2-(2′: 4′-Dimethylpiperidinomethyl)-4: 5-dimethylphenol	*a*	b.p. 147/0·5 mm. n_D^{20} 1·527	HCl: 180–182
0615	1-Piperidinomethyl-3-ethoxycarbonyl-2-naphthol	*a*	m.p. 106–107	M: 121–123
0624	6-Piperidinomethyl-2: 3-dimethylphenol (iv)	*a*	b.p. 128–130/0·5 mm. n_D^{20} 1·537	HCl: 220–221·5
0625	4-Piperidinomethyl-2: 5-dimethylphenol (ii)	*a*	(1, 2)	HCl: 226–227
0626	2-(4′-Ethylpiperidinomethyl)-4: 5-dimethylphenol	*a*	m.p. 28–30	HCl: 162–164
0627	2-(4′-Methylpiperidinomethyl)-4: 5-dimethylphenol	*a*	m.p. 44–46	HCl: 180–182
0628	2-(4′-Ethoxycarbonylpiperidinomethyl)-4: 5-dimethylphenol	*a*	b.p. 152/5 × 10^{-5} mm. n_D^{20} 1·522	HCl: 164–166
0631	2-(4′-Hydroxymethylpiperidinomethyl)-4: 5-dimethylphenol	*a*	m.p. 75–76	HCl: 180–182
0632	2-(3′-Methylpiperidinomethyl)-4: 5-dimethylphenol	*a*	m.p. 52–54	
0633	2-(2′-Methyl-5′-ethylpiperidinomethyl)-4: 5-dimethylphenol	*a*	m.p. 80–81	
0634	4-Piperidinomethyl-2: 6-dimethylphenol	*a*	(1)	M: 135–136
0635	2-Piperidinomethyl-4: 6-dimethylphenol	*a*	(1)	M: 90
0636	6-Piperidinomethyl-5-hydroxyindane (v)	*a*	b.p. 125–126/0·22 mm. n_D^{20} 1·549 (8)	HCl: 206–208 M: 118
0663	1-(α-Piperidinoethyl)-2-naphthol	*e*		T: 125
0670	6-(2′-Methylpiperidinomethyl)-5-hydroxyindane (vi)	*a*	m.p. 35–37 (8)	HCl: 173–175 M: 152–154
0674	3-Piperidinomethyl-2-naphthol	*f*	m.p. 159–160	HCl: 217·5–219·5
0675	2-Piperidinomethyl-3: 4: 6-trimethylphenol	*a*	(1)	HCl: 228–230
0676	2-Piperidinomethyl-4-methylphenol	*a*	(1, 2, 4, 5)	HCl: 198
0677	2-Piperidinomethyl-5-methylphenol	*a*	(1, 2, 4, 5)	HCl: 166–168
0679	(+)-2-(2′-Methylpiperidinomethyl)-4: 5-dimethylphenol	*a*	b.p. 121/0·3 mm. n_D^{20} 1·534 $[\alpha]_D^{20}$ +47·1° (c = 0·98 in benzene)	
0682	2-Piperidinomethyl-3: 4-dimethylphenol	*g*	b.p. 120–122/0·18 mm. n_D^{20} 1·539	HCl: 168–170
0685	2-Hexamethyleneiminomethyl-4: 5-dimethylphenol	*a*	m.p. 52	HCl: 174
0694	(−)-2-(2′-Methylpiperidinomethyl)-4: 5-dimethylphenol	*a*	b.p. 112/0·14 mm. n_D^{20} 1·534 α_D^{20} −51·4° (c = 1·33 in benzene)	
0696	2-Piperidinomethyl-4-chlorophenol	*a*	(5, 6)	HCl: 231
0698	2-Piperidinomethyl-4-chloro-5-methylphenol	*a*	(6)	HCl: 207

TABLE IX—*continued*

Ro 3– (1)	Name (2)	Method of Preparation (3)	Refs. (Known Bases) Phys. Consts. °C. (New Bases) (4)	Salt with M.P. °C. (5)
0699	2-(N-ethyl-N-*iso*propylaminomethyl)-4: 5-dimethylphenol	*a*	b.p. 90/0·15 mm. n_D^{20} 1·516	HCl: 201
0701	2-Isopropylaminomethyl-4: 5-dimethylphenol	*a*	m.p. 75	HCl: 137
0702	2-Diethylaminomethyl-4: 5-dimethylphenol	*a*	(6)	HCl: 190–192
0707	2-Piperidinomethyl-4-ethyl-5-methylphenol	*a*	b.p. 120/0·1 mm. n_D^{20} 1·534	HCl: 160–162
0710	2-Morpholinomethyl-4: 5-dimethylphenol	*a*	b.p. 129/0·4 mm. (9)	HCl: 198
0711	2-Piperidinomethyl-3: 4: 5-trimethylphenol	*a*	m.p. 102–103	HCl: 211
0712	2-(2′-Ethylpiperidinomethyl)-4: 5-dimethylphenol	*a*	m.p. *ca.* 39	HCl: 174–176
0713	2-(2′-Methylpiperidinomethyl)-4-ethyl-5-methylphenol	*a*	b.p. 143/0·5 mm. n_D^{20} 1·532	HCl: 143–145
0722	2-(5′-Ethoxycarbonyl-2′-methylpiperidinomethyl)-4: 5-dimethylphenol	*a*		HCl: 189
0724	2-Piperidinomethyl-4: 5-dimethoxyphenol	*a*	b.p. 119/5 × 10^{-5} mm.	HCl: 170–172
0733	2-Piperidinomethyl-4: 5-diethylphenol	*a*	b.p. 136–138/0·1 mm.	HCl: 178
0734	2-Piperidinomethyl-5-methyl-4-propylphenol	*a*	b.p. 132/0·1 mm. n_D^{20} 1·531	
0737	2-Piperidinomethylphenol	*h*	b.p. 100/0·25 mm. n_D^{20} 1·537	HCl: 160–162
0738	2-(3′-Methylmorpholinomethyl)-4: 5-dimethylphenol	*a*	m.p. 59–61 (9)	HCl: 165–166 M: 162–164
0739	2-Piperidinomethyl-5-ethyl-4-methylphenol	*a*	b.p. 117/0·1 mm.	HCl: 154
0742	2-(β-Morpholinoethyl)-4: 5-dimethylphenol	*i*		HCl: 238–239
0744	2-(β-Piperidinoethyl)-4: 5-dimethylphenol	*j*		HCl: 193–195
0745	6-Morpholinomethyl-5-hydroxyindane (v)	*a*	m.p. 41–44 (8)	M: 133
0747	6-(3′-Methylmorpholinomethyl)-5-hydroxyindane (v)	*a*	m.p. 58–60 (8)	HCl: 193–195 M: 153
0748	2-Piperidinomethyl-4-propylphenol	*a*	b.p. 141/0·75 mm. n_D^{20} 1·528	HCl: 178–180
0752	2-(2′-Methylpiperidinomethyl)-5-ethyl-4-methylphenol	*a*	b.p. 126/0·3 mm. n_D^{20} 1·531	
0754	3-(β-Piperidinoethyl)-indole		(7)	HCl: 222.5–224·5
0760	2-Diallylaminomethyl-4: 5-dimethylphenol	*a*	b.p. 120/0·1 mm. n_D^{20} 1·529	HCl: 136–137
0761	2-Piperidinomethyl-4-cyclohexylphenol	*a*	m.p. 59–60	
0765	(−)-2-(2′-Methylpiperidinomethyl)-4-ethyl-5-methylphenol	*a*	b.p. 126 8/0·19 mm. n_D^{2} 1·530 $[\alpha]_D^{20}$ −45·7° (c = 1·25 in benzene)	
0771	(+)-2-(2′-Methylpiperidinomethyl)-4-ethyl-5-methylphenol	*a*	b.p. 126–8/0·19 mm. n_D^{20} 1·530 $[\alpha]_D^{20}$ +44·4° (c = 1·31 in benzene)	
0777	2-Piperidinomethyl-4-isopropyl-5-methylphenol	*a*	b.p. 122/0·25 mm. n_D^{20} 1·531	
0820	2-Piperidino-4: 5-dimethylphenol	*k*	b.p. 95–97/0·1 mm. n_D^{20} 1·539	
0857	(−)-6-(2′-Methylpiperidinomethyl)-5-hydroxyindane	*a*	b.p. 133–134/0·1 mm. n_D^{20} 1·549 $[\alpha]_D^{20}$ −47·2° (c = 0·68 in benzene)	M: 147–149 $[\alpha]_D^{20}$ −9·9° (c = 1·7 in water)
0858	(+)-6-(2′-Methylpiperidinomethyl)-5-hydroxyindane	*a*	b.p. 136–138/0·12 mm. n_D^{20} 1·549 $[\alpha]_D^{20}$ +44·9° (c = 1·20 in benzene)	M: 144–147 $[\alpha]_D^{20}$ +7·0° (c = 1·63 in water)
0859	2-Dimethylaminomethyl-4: 5-dimethylphenol	*a*	m.p. 80–81	
2–6428	2-Pyrrolidinomethyl-4: 5-dimethylphenol	*a*	b.p. 130/0·1 mm. n_D^{20} 1·538	HCl: 149
0862	1-Acetoxy-2-(2′-methylpiperidinomethyl)-4: 5-dimethylbenzene	*l*	b.p. 118/0·05 mm. n_D^{20} 1·527	
0867	1-Benzoyloxy-2-(2′-methylpiperidinomethyl)-4: 5-dimethylbenzene	*m*	m.p. 77–78	
0885	5-Methoxy-6-(2′-methylpiperidinomethyl)-indane	*n*	b.p. 129–131/0·05 mm. n_D^{20} 1·543	

metrine, but their figures show that the most active are also those with an oxygen function in the *ortho*-position.

Chemical Section

The known and new compounds studied were usually prepared from the appropriate phenol, base and formalin (Mannich reaction) as described in method *a*; other methods of preparation, also described below, are indicated after the name in Table IX, which also lists the properties of new bases and salts, references to literature on known bases, patent references and other details of chemical interest. Satisfactory micro-analyses were obtained for all new bases and salts. Melting points and boiling points are uncorrected.

Methods of Preparation

a.—Equivalent amounts of the phenol, base and formalin were kept overnight in alcoholic solution at room temperature, refluxed for a short period and freed from alcohol under reduced pressure. The residue was dissolved in ether, shaken with *N* sulphuric acid, and, after removal of the ether layer, the acid solution was neutralized with bicarbonate; the product was extracted with ether and isolated by crystallization or vacuum distillation. The hydrochlorides were obtained by treatment of the bases with anhydrous ethereal hydrogen chloride and crystallized from ethanol-anhydrous ether. The maleates and tartrates were obtained by treatment of the bases with molecular equivalents of the acid in alcoholic solution and precipitation with anhydrous ether; they were recrystallized from a mixture of ethanol and anhydrous ether.

b.—The phenol was refluxed in toluene with N-ethoxymethyl-pipecoline, and the product worked up as in method (*a*).

c.—1-Bromo-5 : 6 : 7 : 8-tetrahydro-2-naphthol yielded, in a Mannich reaction (method *a*), 1-bromo-3-piperidinomethyl-5 : 6 : 7 : 8-tetrahydro-2-naphthol from which bromine was eliminated by hydrogenation in acetic acid solution with a palladized barium sulphate catalyst in the presence of potassium acetate.

d.—The known 2-hydroxy-5 : 6 : 7 : 8-tetrahydro-3-naphthoic ester was converted to the *piperidide*, m.p. 202 to 204° C. (see *f*). This was reduced by lithium aluminium hydride to the product (Ro 3-0542) of known structure. This was identical with the products obtained by methods *a* and *c* from 5 : 6 : 7 : 8-tetrahydro-2-naphthol.

e.—The method of preparation was similar to *a*, but acetaldehyde was used in place of formaldehyde.

f.—By boiling 3-ethoxycarbonyl-2-naphthol with piperidine, *2-hydroxy-3-naphthopiperidide* was obtained as colourless prisms, m.p. 229 to 230° C., from methanol. Reduction with lithium aluminium hydride in ether-dioxane gave the base Ro 3-0674.

g.—2-Bromo-4 : 5-dimethylphenol yielded, in a Mannich reaction (method *a*), *2-bromo-4 : 5-dimethyl-6-piperidinomethylphenol*, m.p. 93 to 95° C. Debromination of this intermediate was effected as in method *c*.

h.—A mixture of salicylaldehyde and piperidine was hydrogenated (Pd-C catalyst) and the product worked up as in method *a*.

i.—The Kindler-Willgerodt reaction with 2-benzyloxy-4 : 5-dimethylacetophenone yielded a substituted *phenylacetothiomorpholide*, m.p. 129° C., which was desulphurized with Raney nickel. The resulting base *1-β[(2-benzyloxy-4 : 5-dimethylphenyl)ethyl] morpholine* formed a *picrate*, m.p. 178 to 178.5° C. Hydrogenation of the crude base hydrochloride with Pd-C catalyst yielded Ro 3-0742.

j.—This was prepared as in method *i* through the corresponding phenylacetothiopiperidide. The *hydrochloride* of the intermediate benzyl ether of Ro 3-0744 had m.p. 180 to 181° C.

k.—2-Amino-4 : 5-dimethylphenol reacted with 1 : 5-dibrompentane in boiling butanol in the presence of potassium carbonate. The basic product was worked up as in method *a*.

l.—Ro 3-0594 (free base) was acetylated with acetyl chloride in dry pyridine solution for 20 hr. at 20° C. After removal of solvent at reduced pressure, the basic product was obtained by treatment with cold bicarbonate solution and extraction with ether.

m.—As in *l*, using benzoyl chloride.

n.—A mixture of 5-methoxyindane-6-aldehyde and α-pipecoline was hydrogenated over palladium-charcoal, after which the basic product was isolated as in method *a*.

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References

Akkerman, A. M., de Jongh, D. K., and Veldstra, H. (1951). *Rec. Trav. chim. Pays-Bas*, **70**, 899.
—— and Veldstra, H. (1954). Ibid., **73**, 629.
Arnold, Z., and Hejno, K. (1955). *Chem. Listy*, **49**, 111.
Auwers, K., and Dombrowski, A. (1906). *Leibigs Ann.*, **344**, 280.
Baltzly, R., Dvorkovitz, V., and Phillips, A. P. (1949). *J. Amer. chem. Soc.*, **71**, 1162.
Bell, G. H., and Robson, J. M. (1936). *J. Physiol.*, **88**, 312.
Bovet-Nitti, F. (1952). *R.C. ist. sup. Sanit.*, **15**, 953; 989.
—— and Bovet, D. (1954). *Arch. int. Pharmacodyn.*, **96**, 327.
Brown, G. L., and Dale, H. H. (1935). *Proc. roy. Soc.*, **118***B*, 446.
Burckhalter, J. H., Tendick, F. H., Jones, E. M., Holcomb, W. F., and Rawlins, A. L. (1946). *J. Amer. Chem. Soc.*, **68**, 1894.
Burn, J. H. (1937). *Biological Standardisation*, 1st ed., p. 57. London: Oxford University Press.
Caldwell, W. T., and Thompson, T. R. (1939). *J. Amer. chem. Soc.*, **61**, 2354.

Cohen, A., and Heath-Brown, B. (1954a), Brit. Pat. Appl., 28,318/54; (1954b), ibid., 29,125/54.
Dawes, G. S. (1951). *J. Physiol.*, **112**, 29P.
—— (1952). *Brit. med. Bull.*, **8**, 324.
Fieser, L. F., and Lothrop, W. C. (1936). *J. Amer. chem. Soc.*, **58**, 2050.
Gearien, J. E., and Liska, K. J. (1954). Ibid., **76**, 3554.
Hejno, K., and Arnold, Z. (1953). *Chem. Listy*, **47**, 601.
Hoffmann, K., and Schellenberg, H. (1944). *Helv. Chim. acta*, **27**, 1782.
Hsu, Y.-C. (1948). *Quart. J. Pharm.*, **21**, 146.
De Jongh, D. K., and van Proosdij-Hartzema, E. G. (1952). *J. Pharmacol.*, **105**, 130.
Julia, M. (1955). *Bull. Soc. chim. Fr.*, 830.
Konzett, H., and Rössler, R. (1940). *Arch. exp. Path. Pharmak.*, **195**, 71.
Kraushaar, A. (1954). *Arzneimittel Forsch.*, **4**, 273.
Marini-Bettòlo, G. B., Chiavarelli, S., and Bovet, D. (1950). *Gazz. chim. ital.*, **80**, 281.
—— —— and Bovet-Nitti, F. (1951). Ibid., **81**, 587.
Paton, W. D. M. (1949). *J. Physiol.*, **108**, 57P.
Plieninger, H. (1953). *Chem. Ber.*, **86**, 25.
Pohland, A. (1949). U.S. Pat. 2,589,205; *Chem. Abstr.*, 1952, **46**, 10210.
van Proosdij-Hartzema, E. G., and de Jongh, D. K. (1954). *Arch. int. Pharmacodyn.*, **98**, 335.
Rosen, H., Blumenthal, A., Townsend, P. N., Tislow, R., and Seifter, J. (1956). *J. Pharmacol.*, **117**, 488.
Rothlin, E. (1935). *C.R. Soc. Biol., Paris*, **119**, 1302.
Schindler, O., and Voegtli, W. (1949). *Pharm. Acta Helvet*, **24**, 207.
Tseou, H.-F., and Yang, C.-T. (1939). *J. org. Chem.*, **4**, 123.

Brit. J. Pharmacol. (1957), **12**, 209.

SOME PHARMACOLOGICAL ACTIONS OF FOUR SYNTHETIC ANALOGUES OF OXYTOCIN

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Four structural analogues of oxytocin were investigated with regard to their oxytocic, milk-ejecting, pressor and diuretic/antidiuretic effects. In three of them the isoleucyl group of oxytocin was replaced by a phenylalanyl, leucyl, or valyl residue; in the fourth the asparaginyl group was replaced by a glutaminyl residue. Synthetic oxytocin and the international standard pituitary (posterior lobe) powder were used for comparison. Although the analogues showed marked differences in their oxytocic effects, there was a fairly good agreement between the results obtained on the isolated rat uterus and the blood pressure of the chicken for each polypeptide. The milk-ejection pressure test gave much higher values throughout. The pressor and antidiuretic activities of the four analogues showed no obvious correlation with the values obtained in the other tests. The valyl and the leucyl analogues also had a diuretic effect. The *phenylalanyl* analogue was remarkable for the close correspondence between its oxytocic and antidiuretic effects: practically identical values were obtained for the potency, whether measured on the rat uterus *in vitro*, the blood pressure in the chicken, the cat uterus *in situ* or water diuresis in the rat. The *leucyl* analogue showed an oxytocic activity on the cat uterus *in situ* or the rabbit mammary gland roughly 7 to 9 times as high as that measured by means of the conventional bioassay methods, such as the blood pressure of the chicken or the rat uterus *in vitro*. The *glutaminyl* analogue, the weakest of the whole series, had only a modest effect on the mammary gland. The *valyl* analogue was the most interesting of the new polypeptides. Its oxytocic action on the cat uterus *in situ* and its milk-ejecting effect were greater than that of synthetic oxytocin, whereas its antidiuretic and pressor effects were less. In cats and rats, the uterine effect was stronger *in situ* than *in vitro*. There were also distinct species differences between cats, rabbits, and rats in their sensitivity to valyl-oxytocin.

Methods recently developed for the synthesis of oxytocin (du Vigneaud, Ressler, Swan, Roberts, Katsoyannis, and Gordon, 1953; Boissonnas, Guttmann, Jaquenoud, and Waller, 1955) have not only made available preparations of this hormone containing no vasopressin, but also permitted the synthesis of structural analogues of oxytocin. A series of such cyclic octapeptides (Table I) has been prepared by Boissonnas, Guttmann, Jaquenoud, and Waller (1956a), the isoleucyl group of oxytocin being replaced by a phenylalanyl, leucyl or valyl group. The valyl derivative (" valyl-oxytocin ") proved to be the most active of these three analogues of oxytocin. Some of its properties have already been briefly described (Boissonnas, Guttmann, Jaquenoud, Waller, Konzett, and Berde, 1956b). A fourth analogue of oxytocin has been obtained by replacing the asparaginyl group by a glutaminyl group (Boissonnas, Guttmann, Jaquenoud, and Waller, 1956a).

In the meantime, Rudinger, Honzl, and Zaoral (1956), in a preliminary communication, reported the synthesis of two analogues of oxytocin having a leucyl or valyl group instead of the isoleucyl group; and du Vigneaud (personal communication) prepared an analogue of oxytocin having a phenylalanyl group instead of the isoleucyl group (" oxypressin ").

The investigations described below are concerned with the effects of the four analogues of oxytocin prepared by Boissonnas, Guttmann, Jaquenoud, and Waller (1956a). For simplicity, these will be referred to as the phenylalanyl (P), leucyl (L), valyl (V) and glutaminyl (G) analogues of oxytocin (Table I). A synthetic preparation of oxytocin (Syntocinon, Sandoz), described by Boissonnas, Guttmann, Jaquenoud, and Waller

TABLE I
THE SEQUENCE OF THE AMINO ACIDS IN OXYTOCIN AND ITS ANALOGUES

Synthetic oxytocin	CyS—Tyr—Ileu—Glu(NH_2)—Asp(NH_2)—CyS—Pro—Leu—Gly(NH_2)
P-analogue	CyS—Tyr—*Phe*—Glu(NH_2)—Asp(NH_2)—CyS—Pro—Leu—Gly(NH_2)
L-analogue	CyS—Tyr—*Leu*—Glu(NH_2)—Asp(NH_2)—CyS—Pro—Leu—Gly(NH_2)
V-analogue (" valyl-oxytocin ") ..	CyS—Tyr—*Val*—Glu(NH_2)—Asp(NH_2)—CyS—Pro—Leu—Gly(NH_2)
G-analogue	CyS—Tyr—Ileu—Glu(NH_2)—*Glu*(NH_2)—CyS—Pro—Leu—Gly(NH_2)

(1955) and Konzett, Berde, and Cerletti (1956), and the international standard pituitary (posterior lobe) powder were used for comparison.

These polypeptides were first tested on the isolated rat uterus and on blood pressure in the chicken, both being methods recommended in pharmacopoeias for oxytocin assay, and on the milk-ejection pressure of the rabbit mammary gland. All three assay methods yielded quantitative data. However, it became evident that further tests were necessary to distinguish between these compounds. The study was therefore extended to include investigations on the cat uterus *in situ* and *in vitro*, on the rat uterus *in situ*, on the induction of labour in non-anaesthetized pregnant rabbits, on the blood pressure of spinal cats and on urine secretion in non-anaesthetized rats. Quantitative results with well-defined limits of error were provided by many of these methods, for example the cat uterus *in situ* and *in vitro*, the induction of labour in non-anaesthetized rabbits and the antidiuresis in non-anaesthetized rats. The other methods, such as the rat uterus *in situ*, the blood pressure in spinal cats and the diuresis in non-anaesthetized rats, provided a rough measure of the activity, and more information on the type of action, of these polypeptides.

METHODS

Solutions.—To obtain the cyclic octapeptides, 20 mg. of the corresponding nonapeptide (Boissonnas, Guttmann, Jaquenoud, and Waller, 1956a) was dissolved in 5 ml. liquid ammonia and reduced with sodium (du Vigneaud, Ressler, Swan, Roberts, and Katsoyannis, 1954). Ammonium chloride (5 mg.) was added; the ammonia was evaporated and the residue added to 100 ml. of water, the *p*H adjusted to 6.8, CO_2-free air bubbled through the solution for 2 hr., and the *p*H adjusted to 4.5. Saline was used for further dilution.

Isolated Rat Uterus.—On the day preceding the experiment the rats were treated with 0.2 mg. stilboestrol subcutaneously to increase the sensitivity of the uterus. The assays and the statistical treatment of the results were carried out according to the method of Holton (1948).

Chicken Blood Pressure.—The method of Coon (1939), using white Leghorn roosters, was followed; for the statistical analysis the procedure of Thompson (1944) was utilized.

Milk-ejection Pressure of the Rabbit Mammary Gland.—The pressure within the lactating mammary gland of rabbits was measured by the method of van Dyke, Adamsons and Engel (1955), modified according to Berde and Cerletti (1956b). The tests were made between the 3rd and 27th day *post partum*. The anaesthetics used were urethane (1.25 g./kg., subcutaneously) with pentobarbitone (10 mg., intravenously) given as a supplement to urethane whenever necessary. Artificial respiration was provided in many experiments but not in all. One of the ducts in a nipple was cannulated and connected to a Statham strain-gauge manometer, the milk-ejection pressure being recorded on a photokymograph. After preliminary trials, the final test was carried out as a four-point assay (Schild, 1942), the doses of the standard and of the unknown substance being injected intravenously at intervals of 3 min.

Rat Uterus in situ.—Rats treated with stilboestrol (0.2 mg., subcutaneously) on each of 2 days preceding the experiment were anaesthetized with urethane (1.4 g./kg., subcutaneously). A midline incision was made and one horn of the uterus was attached to a string by means of a suture through the connective tissue near the end of the horn. The string was led over a pulley and attached to the short arm of a frontal writing lever which recorded the contractions of the uterus on a smoked drum (magnification × 2). To prevent the horn of the uterus from drying, it was covered by loosely suturing the skin which had been divided by the midline incision. Injections were made into the jugular vein at intervals of 60 min. The reactions to identical doses of the international pituitary standard (0.05 to 0.2 *I.U.*) often varied greatly in the same experiment, but in good preparations the response remained fairly constant. A double dose elicited a much stronger contraction. Various doses

of the unknown preparation and a constant dose of the standard were given alternately. The effectiveness of the unknown preparatio was estimated by "bracketing" doses of the unknown with doses of the standard. The results obtained did not permit an accurate determination of the limits of error.

Cat Uterus in situ.—Non-pregnant animals were anaesthetized with a mixture of urethane (0.428 g./kg.) and chloralose (0.0428 g./kg., subcutaneously). Most of them received no pretreatment; a few were given α-oestradiol and progesterone, as recommended by Clary, Cameron, and Craver (1951). The uterus was exposed by a median incision. A thread was looped round one of the uterine horns and attached to a lever fitted with a frontal writing lever (magnification ×3). Here again the tests were based on the technique of "bracketing" constant doses of the standard with differing doses of the unknown preparation. The activity of the unknown substance and the approximate standard error were estimated by the method of Miller and Tainter (1944).

Cat Uterus in vitro.—Uterine segments (1 to 2 cm. in length) of non-pregnant animals were suspended in a 50 ml. bath containing oxygenated Tyrode solution with 0.1% glucose, and attached to a lever weighing 1 to 5 g. (magnification ×3). Otherwise these tests were carried out in the same way as those on the cat uterus *in situ*.

Induction of Labour in the Rabbit.—It is known that pregnant rabbits near to term frequently go into labour if posterior pituitary extract (Knaus, 1926) or oxytocin (Csapo, 1955) is injected. Non-anaesthetized rabbits were used on the morning of the 31st day of pregnancy (exactly 720 hr. after mating). The substances to be tested were injected intramuscularly. The induction of labour was considered successful if the first foetus was delivered within 60 min. of the injection; as a rule, this happened within a few minutes if the dose administered was effective. By contrast, only 1 of 25 control animals injected with saline solution delivered within 1 hr. Three doses of each substance were tested, each dose being given to a group of 20 or 25 rabbits. The logarithm of the dose was plotted against the probit effect in order to determine the ED50 and its approximate standard error, as described by Miller and Tainter (1944).

In addition to these estimations of oxytocic activity, further investigations were performed to estimate the effect of the polypeptides on blood pressure and urine secretion.

Pressor Activity.—Spinal cats were used. Here again the technique of "bracketing" doses of the unknown substance with doses of the standard was used. The results obtained did not permit accurate determination of the limits of error.

Effect on Urine Secretion.—A slightly modified form of the water diuresis test of Burn (1931, 1950) was carried out on non-anaesthetized male rats weighing 130 to 200 g. On the evening preceding the experiment all food was removed from the cage. The following morning the animals received 2.5 ml./100 g. tap-water at body temperature by stomach tube. A loading dose of 5 ml./100 g. of water was given by stomach tube 2½ hr. later, when the test preparation was injected subcutaneously. In all other respects the experiment and the calculation of the time of maximum rate of excretion were carried out according to Burn's recommendations. The results gave a measure of the antidiuretic effect. Once the approximate activity of a substance had been established the final test was carried out as a four-point assay (Schild, 1942).

The relation between the loading amount of water and the total quantity of urine excreted during the period of enhanced diuresis was calculated in order to detect a possible diuretic effect (Fraser, 1937).

RESULTS

Table II summarizes most of the results obtained with synthetic oxytocin and its four synthetic analogues. Identical values were obtained for the activity of synthetic oxytocin when it was assayed on the isolated rat uterus, on blood pressure in the chicken or on the milk-ejection pressure of the rabbit mammary gland. When tested on the cat uterus *in situ* an activity about 30% higher than that obtained with other methods was found. A certain amount of antidiuretic and pressor activity was inherent in synthetic oxytocin, but it was only equivalent to about 1% of the oxytocic potency.

TABLE II

ACTIVITY OF 1 ML. OF SOLUTIONS OF THE CYCLIC OCTAPEPTIDES IN UNITS OF INTERNATIONAL STANDARD PITUITARY (POSTERIOR LOBE) POWDER IN DIFFERENT TESTS

	Isolated Rat Uterus	Chicken Blood Pressure	Milk-ejection Pressure (Rabbit Mammary Gland)	Cat Uterus *in situ*	Antidiuretic Activity (Non-anaesthetized Rats)	Pressor Activity (Spinal Cats)
Synthetic oxytocin	8·0 (±0·22)	8·1 (±0·16)	8·3 (±0·23)	10·7 (±0·9)	0·09 (±0·05)	0·07
P-analogue	2·7 (±0·11)	2·2 (±0·08)	5·8 (±0·37)	2·4 (±0·3)	2·8 (±1·2)	0·5
L-analogue	0·25 (±0·01)	0·33 (±0·01)	2·2 (±0·44)	2·3 (±0·4)	0·15 (±0·08)	0·15
V-analogue ("valyl-oxytocin")	2·8 (±0·11)	3·2 (±0·08)	15·0 (±0·75)	16·7 (±1·3)	0·04 (±0·02)	0·015
G-analogue	<0·0025	<0·02	0·025 (±0·003)	0 (up to 1 ml./kg. i.v.)	0 (up to 1 ml./100 g. s.c.)	0 (up to 0·5 ml./kg. i.v.)

There was a fairly good correlation between the effect of each analogue on the isolated rat uterus and the chicken blood pressure, but the effects in the milk-ejection pressure test were greater. This was true even of the G-analogue, which had practically no detectable effect on the isolated rat uterus and chicken blood pressure but showed some effect on milk-ejection pressure. The effect of the four compounds on the cat uterus *in situ* followed a different pattern. Whereas the L- and V-analogues exhibited comparable effects on the milk-ejection pressure and on the cat uterus *in situ* (with a tendency for the effect on the latter to be slightly increased) the P- and G-analogues were less active on the cat uterus *in situ* than on the milk-ejection pressure.

The pressor and antidiuretic activities of the four analogues bore no obvious relation to the values obtained in the other tests. The P-analogue had a marked pressor activity and a remarkably great antidiuretic effect whereas the other compounds are much less active. If the total quantity of urine excreted in Burn's test during the period of enhanced diuresis was taken into consideration, a characteristic pattern of reaction to three of the synthetic polypeptides was revealed. The rats treated with 0.016–0.5 ml./100 g. synthetic oxytocin, 0.1 and 0.2 ml./100 g. valyl-oxytocin or 0.03 and 0.06 ml./100 g. of the L-analogue excreted significantly more urine than those treated with various doses of the international standard powder or the controls which received 0.2 ml./100 g. saline. No such diuretic reaction was seen after the administration of the P- and G-analogues.

The most interesting of the new polypeptides was valyl-oxytocin. It had an effect greater than that of synthetic oxytocin on milk-ejection pressure and on the cat uterus *in situ*. The antidiuretic and the pressor effects, however, were weaker than those of synthetic oxytocin. Further investigation of this compound on the cat uterus *in vitro* and on the rat uterus *in situ* revealed that, in both species its effect on the uterus *in situ* was much greater than on the uterus *in vitro*. The potency of valyl-oxytocin on the isolated rat uterus was 2.8 ($\pm$0.11) *I.U.*/ml., and on the rat uterus *in situ* about 10 *I.U.*/ml. The values for the isolated cat uterus were 6 ($\pm$0.6) *I.U.*/ml. and for the cat uterus *in situ* 16.7 ($\pm$1.3) *I.U.*/ml. Although the results obtained in the studies on the rat uterus *in situ* did not permit a statistical analysis to be made, the difference between the oxytocic action of valyl-oxytocin *in vitro* and *in situ* existed in both species.

The activity of valyl-oxytocin in inducing labour in rabbits near term was found to be equivalent to 6.2 ($\pm$3) *I.U.*/ml. and that of synthetic oxytocin to be 9.3 ($\pm$4.1) *I.U.*/ml.

Discussion

The present investigations on some pharmacological actions of synthetic oxytocin and four synthetic oxytocin analogues show that the substitution of one amino acid by another in the oxytocin molecule resulted in cyclic polypeptides with somewhat different effects. This is not surprising in view of the fact that replacement of two amino acids in the oxytocin molecule by two others (replacing isoleucine by phenylalanine, and leucine by arginine or lysine) yields vasopressin (du Vigneaud, Lawler, and Popenoe, 1953).

The P-analogue, in which the phenylalanyl group is in the same position as in the bovine and the porcine types of vasopressin, and which therefore has a cyclic part identical with that of vasopressin, exhibits a weaker oxytocic effect than oxytocin and enhanced antidiuretic and pressor effects. The antidiuretic and pressor activity of the P-analogue is greater than that of the other preparations in the series. There is, however, a discrepancy between the potency as measured by the antidiuretic effect and that measured by the pressor effect of the P-analogue. This seems to be an indication of the relative importance of arginine and lysine for the pressor effect of vasopressin. In the milk-ejection pressure test, the P-analogue showed a remarkable potency of more than twice that measured on the isolated rat uterus, the chicken blood pressure and the cat uterus *in situ*.

Du Vigneaud's highly purified P-analogue ("oxypressin") also showed its maximum activity in the milk-ejection pressure test (du Vigneaud, personal communication). As the details of the biological investigation with du Vigneaud's oxypressin are not yet available, a quantitative comparison between the potencies of oxypressin and our P-analogue in different tests would be premature.

With regard to the L-analogue, it is surprising to find that the oxytocic effect on the cat uterus *in situ* is about 9 times as great as that on the rat uterus *in vitro*. The pronounced effect on the cat uterus *in situ* is in accord with the effect on milk-ejection pressure. The antidiuretic activity of the L-analogue, however, is remarkable. The ratio between antidiuretic effect and oxytocic effect (on the isolated rat uterus or on the cat uterus *in vivo*)

is definitely greater for the L-analogue than for synthetic oxytocin.

The G-analogue exhibits such a low activity that definite values could only be obtained in the milk-ejection pressure test. The substitution of glutamic acid for aspartic acid results in far greater changes in activity than is obtained by replacing the isoleucyl group by other amino acids.

The V-analogue, which we (Boissonnas, Guttmann, Jaquenoud, Waller, Konzett, and Berde, 1956b) suggested be termed " valyl-oxytocin," is of great interest because of its high oxytocic potency *in vivo* and its low vasopressor and antidiuretic activity. If the values obtained in studies on the cat and rat uterus *in situ*, the induction of labour in rabbits and the milk-ejection pressure test in the rabbit are compared with those obtained for water diuresis in non-anaesthetized rats and blood pressure in spinal cats, the ratio of the oxytocic to the pressor-antidiuretic potency is greater for valyl-oxytocin than for oxytocin.

In cats and rats there is a marked discrepancy between the effect of valyl-oxytocin on the uterus *in situ* and *in vitro*. It is not yet possible to give an explanation for this phenomenon, especially as synthetic oxytocin and the P-analogue do not exhibit the same difference in activity on the uterus *in vitro* and *in situ*.

Rudinger, Honzl, and Zaoral (1956) in their preliminary communication state that their L- and V-analogues of oxytocin " showed uterus-contracting, avian vasodepressor and milk-ejecting activities lower by about one order of magnitude than the corresponding activities of oxytocin." They do not mention the marked effect of the valyl analogue of oxytocin on milk-ejection pressure. The same authors say that " the antidiuretic effect of the valine analogue was about equal to that of the natural hormone, the activity of the leucine derivative some ten times greater." Since the methods used in these studies are not given in detail, no comment can be made on the discrepancy between their findings and ours.

It is remarkable that synthetic oxytocin and its analogues exhibit a very high degree of activity in the milk-ejection pressure test. This method has already been described as highly sensitive for oxytocin (van Dyke, Adamsons, and Engel, 1955). It seems that the myoepithelial cells of the rabbit mammary gland, which bring about the increase in the milk-ejection pressure (Linzell, 1955), are also highly sensitive to all the synthetic analogues of oxytocin included in this study.

The diuretic action of synthetic oxytocin and two of its analogues (valyl-oxytocin and the L-analogue) deserves some comment. Fraser (1937) first observed that posterior pituitary extracts containing mainly oxytocin exert a bivalent effect on water diuresis in non-anaesthetized rats: in high doses, such extracts exhibit an antidiuretic activity in that the interval between the oral administration of the loading dose of water and the time of maximum rate of excretion is prolonged. But they also exert a diuretic action in that the total quantity of urine excreted during the period of enhanced diuresis is increased. It has recently been ascertained that both the antidiuretic and diuretic properties are inherent in the oxytocin molecule, for they are also shown by synthetic oxytocin which contains no vasopressin (Berde and Cerletti, 1956a). It is possible, however, that the diuretic activity of oxytocin is confined to one species only. Posterior pituitary extracts containing mainly oxytocin exhibit a diuretic activity in rats, not only in Burn's water diuresis test but also under various other experimental conditions (Fraser, 1937, 1942 ; Kuschinsky and Bundschuh, 1939 ; Schaumann, 1949 ; Brunner, Kuschinsky, Münchow, and Peters, 1956 ; Brunner, Kuschinsky, and Peters, 1956 ; Croxatto, Rosas, Zamorano, and Gonzalez, 1956). No diuretic effect has yet been observed in man (Brunner, Kuschinsky, Münchow, and Peters, 1956) or in dogs (van Dyke, Adamsons, and Engel, 1955), although it has recently been shown that in conscious dogs at resting rates of urine flow oxytocin elicits a marked increase in Na and Cl excretion (Brooks and Pickford, 1956).

The structural analogues of oxytocin included in these investigations possess in common several biological activities but to different degrees. It is evident that to characterize a compound with oxytocin-like properties a large number of tests must be used. The exclusive use of conventional bioassay methods for oxytocin, such as the avian blood pressure and the rat uterus *in vitro*, is not sufficient for this purpose.

In a recent paper, Ressler (1956) showed that the cyclic disulphide ring of oxytocin—deprived of the prolylleucyl-glycinamide side-chain—exhibited some effect on the isolated rat uterus and, to a smaller degree, on the mammary gland, whereas practically no influence on the chicken blood pressure could be detected. These results, like our own, emphasize the need to use a variety of tests to define the biological activities of compounds related to oxytocin.

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References

Berde, B., and Cerletti, A. (1956a). *Helv. physiol. acta*, **14**, 129.

—— —— (1956b). *Journées Franco-Belgo-Suisses de Gynécologie et d'Obstétrique, Bâle.* In the press.

Boissonnas, R. A., Guttmann, St., Jaquenoud, P. A., and Waller, J. P. (1955). *Helv. chim. acta*, **38**, 1491.

—— —— —— —— (1956a). Ibid., **39**, 1421.

—— —— —— —— Konzett, H., and Berde, B. (1956b). *Nature, Lond.*, **178**, 260.

Brooks, F. P., and Pickford, M. (1956). *Abstr. XXth int. physiol. Congr., Bruxelles*, p. 130.

Brunner, H., Kuschinsky, G., Münchow, O., and Peters, G. (1956). *Klin. Wschr.*, **34**, 451.

—— —— and Peters, G. (1956). *Arch. exp. Path. Pharmak.*, **228**, 457.

Burn, J. H. (1931). *Quart. J. Pharm.*, **4**, 517.

—— Finney, D. J., and Goodwin, L. G. (1950). *Biological Standardization*, 2nd ed. Oxford: University Press.

Clary, M. L., Cameron, A., and Craver, B. N. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 778.

Coon, J. M. (1939). *Arch. int. Pharmacodyn.*, **62**, 79.

Croxatto, H., Rosas, R., Zamorano, B., and Gonzalez, I. (1956). *Abstr. XXth int. physiol. Congr., Bruxelles*, p. 205.

Csapo, A. (1955). In *Modern Trends in Obstetrics and Gynaecology* (*Second Series*), p. 20. London: Butterworth.

van Dyke, H. B., Adamsons, K. Jr., and Engel, S. L. (1955). *Recent Progr. Hormone Res.*, **11**, 1.

Fraser, A. M. (1937). *J. Pharmacol.*, **60**, 89.

—— (1942). *J. Physiol.*, **101**, 236.

Holton, P. (1948). *Brit. J. Pharmacol.*, **3**, 328.

Knaus, H. H. (1926). *J. Physiol.*, **61**, 383.

Konzett, H., Berde, B., and Cerletti, A. (1956). *Schweiz. med. Wschr.*, **86**, 226.

Kuschinsky, G., and Bundschuh, H. E. (1939). *Arch. exp. Path. Pharmak.*, **192**, 683.

Linzell, J L. (1955). *J. Physiol.*, **130**, 257.

Miller, L. C., and Tainter, M. L. (1944). *Proc. Soc. exp. Biol., N.Y.*, **57**, 261.

Ressler, C. (1956). Ibid., **92**, 725.

Rudinger, J., Honzl, J., and Zaoral, M. (1956). *Coll. Trav. chim. Tchécosl.*, **21**, 770.

Schaumann, O. (1949). *Experientia*, **5**, 360.

Schild, H. O. (1942). *J. Physiol.*, **101**, 115.

Thompson, R. E. (1944). *J. Pharmacol.*, **80**, 373.

du Vigneaud, V., Lawler, H. C., and Popenoe, E. A. (1953). *J. Amer. chem. Soc.*, **75**, 4880.

—— Ressler, C., Swan, J. M., Roberts, C. W., and Katsoyannis, P. G. (1954). Ibid., **76**, 3115.

—— —— —— —— —— and Gordon, S. (1953). Ibid., **75**, 4879.

Brit. J. Pharmacol. (1957), **12**, 215.

VENTRICULAR FIBRILLATION IN THE ISOLATED RABBIT HEART

BY

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Ventricular fibrillation has been produced in the isolated and perfused rabbit heart by stimulating electrically at a rate from 500 to 700/min. When the perfusion fluid contained normal amounts of sodium, potassium and calcium, the fibrillation persisted after the stimulation was stopped in about 40% of hearts. When the sodium was reduced to half, tonicity being maintained by sucrose or by choline chloride, persistent fibrillation was observed in 100% of hearts. The addition of eserine or of atropine or of carbachol did not alter the percentage of hearts in which fibrillation persisted. The antimalarial compounds chloroquine, mepacrine, and pyrimethamine arrested persistent fibrillation, restoring a normal rhythm.

Ventricular fibrillation can be produced in the Langendorff preparation of the rabbit heart by stimulating the ventricles at a high rate. At the end of stimulation the fibrillation continues in some hearts indefinitely, while in other hearts it stops after a minute or two. By testing a large group of hearts it is possible to determine the proportion of hearts in which fibrillation continues indefinitely. This proportion has been found to depend on the composition of the fluid perfusing the heart, and we have already described the effect of changes in the concentration of potassium ions and of calcium ions (Armitage, Burn and Gunning, 1957).

In this paper we describe the effects of (1) reducing the concentration of sodium ions, (2) adding carbachol, eserine or atropine to the perfusing fluid, and (3) the antimalarial substances chloroquine, mepacrine and pyrimethamine.

METHOD

Rabbit hearts were perfused with a solution recently described by McEwen (1956) containing NaCl 7.7 g., KCl 0.42 g., $CaCl_2$ 0.24 g., $NaHPO_4,2H_2O$ 0.143 g., $NaHCO_3$ 2.1 g., dextrose 2.0 g., sucrose 4.5 g., distilled water 1,000 ml. Thus the Na^+ concentration was 163 mM./l., the K^+ concentration was 5.6 mM./l., and the Ca^{++} concentration was 2.2 mM./l. The solution was saturated with O_2+5% CO_2 before being placed in the apparatus, and aerated again with the same mixture before going to the heart. The *p*H was 7.4. The temperature of the solution entering the heart was maintained constant at 37° C. at all rates of coronary flow by using the device described by Saxby (1956). For stimulation a pair of platinum electrodes was inserted in the ventricles through which rectangular pulses of 1 mA. strength and of 0.75 msec. duration could be applied at varying rates. A second pair of electrodes was inserted in the ventricles on the opposite side as leads to a Cossor electrocardiograph (model 1314). A mechanical record was also taken. When a heart was set up it was perfused for 30 min. with the solution described before changing to a modification of the solution which was to be tested. Stimulation was applied at a rate which was increased until fibrillation began. This usually occurred at a rate between 500 and 700/min. Stimulation was then continued for 5 min. When stimulation ceased the time for which fibrillation persisted was observed.

RESULTS

The Reduction of Sodium.—The solution already described was used to perfuse a series of 28 hearts. When fibrillation was established and stimulation was stopped, the fibrillation persisted during 30 min. in 11 hearts, while in 17 hearts there was spontaneous reversion to normal rhythm within 5 min. The amount of sodium chloride in the perfusing fluid was then reduced to one-half. In 13 experiments the tonicity was maintained by adding sucrose, and in each of these fibrillation continued for 30 min. Since the replacement by sucrose reduced not only sodium but also chloride, other experiments were carried out in which choline chloride was used instead of sucrose. The large amount of choline chloride depressed the rate and amplitude and caused A-V block in some hearts. The first two hearts, in which there was no block,

fibrillated for 30 min. after stimulation, while in the next two hearts block developed and stimulation failed to cause fibrillation. In the remaining five experiments 0.2 mg. atropine sulphate was injected into the cannula every 15 min., and stimulation then caused fibrillation which persisted for 30 min. in each experiment. The results are shown in Table I; they make it evident that the decrease in sodium increased the proportion of hearts fibrillating.

TABLE I

EFFECT OF REDUCTION IN Na^+ CONCENTRATION ON PROPORTION OF HEARTS FIBRILLATING

Na^+ Conc. mM./l.	Tonicity Maintained by Addition of	Proportion of Hearts Fibrillating for 30 min.
163	—	11/28
81·3	Sucrose	13/13
81·5	Choline chloride	7/7

Potassium Efflux.—In the previous experiments (Armitage, Burn and Gunning, 1957) it was observed that the efflux of potassium was increased during fibrillation whether the fibrillation was caused by reducing the potassium or by raising the calcium in the perfusion fluid. The estimation was made by the use of a Beckman flame photometer, the samples of perfusate being collected (1) while the rhythm was normal, (2) while the heart was fibrillating during stimulation, (3) while the heart was fibrillating after stimulation, and (4) after re-establishing normal rhythm by injecting 10 mg. KCl into the cannula. The mean result for samples (2) and (3) was then compared with that for samples (1) and (4).

Estimations of the potassium in the perfusate were made in the same way during perfusion with half the normal amount of sodium, and the results of five experiments are given in Table II. During fibrillation there was again a higher efflux of potassium ions in each experiment, the mean increase being 2.3%.

TABLE II

FIBRILLATION DURING PERFUSION WITH A SOLUTION CONTAINING HALF THE NORMAL SODIUM CONCENTRATION

K^+ Efflux (mg./l.)		
During Fibrillation	Before and After Fibrillation	% Increase During Fibrillation
224	217	3·0
212	211	0·5
226	218	3·7
223	217	2·8
225	222	1·3
Mean 222	217	2·3

Effect of Eserine and Atropine.—In the heart-lung preparation of the dog, electrical stimulation of the atria has been shown to produce auricular fibrillation when eserine was added to the blood (Burn, Vaughan Williams and Walker, 1955). Fibrillation produced in this way or by stimulation during the infusion of acetylcholine was arrested by atropine. Experiments were therefore carried out to see whether the presence of eserine increased the proportion of hearts with ventricular fibrillation and whether the presence of atropine diminished it. The results are given in Table III.

TABLE III

EFFECT OF ESERINE AND ATROPINE ON PROPORTION OF HEARTS FIBRILLATING

	Drug Conc. M.	Conc. of K^+ in Perfusion Fluid mM.	Proportion of Hearts Fibrillating
Control ..	—	5·6	11/28
Eserine ..	$1{\cdot}2\times10^{-6}$	5·6	1/5
Control ..	—	2·8	10/11
Atropine	(See text)	2·8	13/15

In testing the effect of atropine, the solution used to perfuse the hearts contained half the normal potassium concentration, since this solution was previously found to cause fibrillation in 10 out of 11 hearts. In 10 of the experiments, 0.2 mg. atropine sulphate was injected into the cannula every 15 min., while in the other five experiments atropine sulphate was added to the perfusing solution in a concentration which varied from 5×10^{-8} to 10^{-6} g./ml. The results showed that eserine did not increase, and atropine did not diminish, the proportion of hearts fibrillating.

In two experiments the effect of injecting atropine on fibrillation during the stimulation was observed. In the first of these four injections of 0.5 mg. atropine, and in the second six injections of 0.5 mg. atropine arrested the fibrillation. In two other experiments in which the hearts had already fibrillated for more than 30 min. after stimulation, injection of atropine, 2.4 mg. and 1.0 mg. respectively, abolished fibrillation and restored a normal rhythm. These amounts were greatly in excess of that required to "atropinize" the heart.

The Effect of Carbachol.—In six experiments the effect of adding carbachol to the perfusing fluid was determined. The results are shown in Table IV. In none of the experiments did the presence of carbachol increase the tendency to fibrillate. In experiments 1 and 6 in which fibrillation was induced in the control period by driving at rates of 526 and 623 respectively, much higher rates of stimulation were required

TABLE IV

EFFECT OF CARBACHOL ON FIBRILLATION

Perfusion fluid contained 5.6 mM./l. K^+ and 1.1 mM./l. Ca^{++}

Expt.	Carbachol (g./ml.)	Rate of Stimulation to Induce Fibrillation		Duration of Fibrillation	
		Control	Carbachol	Control	Carbachol
1	5×10^{-7}	526	888	2·5 min.	More than 15 min.
2	2×10^{-6}	1,000	1,000	(No fibrillation)	(No fibrillation)
3	2×10^{-7}	1,000	1,000	,, ,,	,, ,,
4	10^{-7}	616	525	More than 15 min.	More than 15 min.
5	2×10^{-6}	1,000	1,000	(No fibrillation)	(No fibrillation)
6	10^{-7}	623	1,064	More than 15 min.	More than 15 min.

to cause fibrillation in the presence of carbachol. In experiments 4 and 6 in which fibrillation persisted in the control period, normal rhythm was restored by rapid cooling of the heart. This method of arresting fibrillation was described by Dirken, Gevers, Heemstra and Huizing (1955).

Effects of Chloroquine, Mepacrine and Pyrimethamine.—Armitage (1957) has observed that chloroquine, mepacrine and pyrimethamine have a quinidine-like action on the electrically-driven rabbit atria (Dawes, 1946). It therefore seemed likely that these substances would be effective in abolishing fibrillation, and their action was tested. Fig. 1 shows the ECG records from three experiments in which ventricular fibrillation had continued for more than 30 min. In the first experiment the fibrillation was arrested by chloroquine; in the second it was arrested by mepacrine, and in the third it was arrested by pyrimethamine. The concentrations of these substances in the perfusion fluid were respectively 2×10^{-5} g./ml., 10^{-5} g./ml., and 2×10^{-5} g./ml.

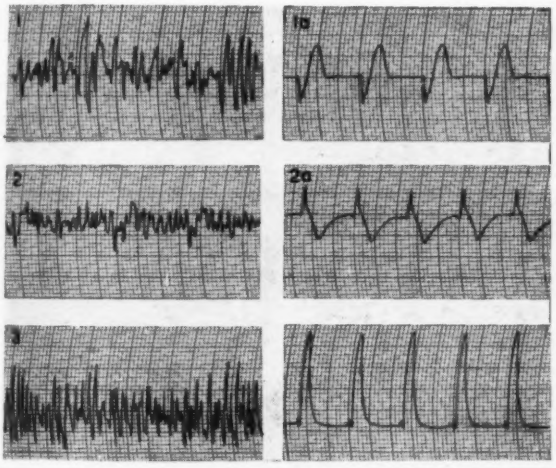

FIG. 1.—ECG records of isolated rabbit heart. (1) Shows ventricular fibrillation which continued for more than 30 min. (1a) Shows the normal rhythm restored within 4 min. by including chloroquine (2×10^{-5} g./ml.) in the perfusion fluid. (2) A different experiment; record as in (1). (2a) Shows the normal rhythm restored within 7 min. by including mepacrine (10^{-5} g./ml.) in the perfusion fluid. (3) A third experiment; record as in (1). (3a) Shows the normal rhythm restored by pyrimethamine (2×10^{-5} g./ml.) in 2 min.

DISCUSSION

The evidence that neither eserine, atropine nor carbachol affected the production of ventricular fibrillation is of interest because Hoffman and Suckling (1953) found that acetylcholine did not modify the ventricular action potential. By contrast these workers found that acetylcholine shortened the atrial action potential, and, since acetylcholine greatly facilitates atrial fibrillation, it appears that fibrillation in the atria or in the ventricles occurs only when the action potential is shortened. We now know that the shortening of the action potential in the atria is accompanied by an increase in the membrane conductance (Trautwein, Kuffler and Edwards, 1956) and by an increased permeability to potassium (Harris and Hutter, 1956).

Evidence is accumulating that in fibrillation there is an increased loss of potassium. In the isolated atria Holland, Burn, and Schümann (1957) have found that stimulation alone at increasing rates does not appreciably increase the potassium loss. If, however, the stimulation is applied in the presence of acetylcholine the potassium loss increases with the rate of stimulation until fibrillation occurs when the potassium loss exceeds a certain value (Holland *et al.*, 1957). Fibrillation in the ventricles is also attended by increased potassium loss, whether the fibrillation is caused by stimulation in a medium low in potassium, high in calcium, or, as has now been shown, low in sodium. The observations on potassium loss in the ventricles are, however, preliminary, and closer study is needed.

It is not easy to understand why a reduction of sodium to half should increase the tendency to fibrillation so greatly. One suggestion which can be made is that, when the sodium is reduced, less sodium enters the cell during contraction, and as a result the sodium pump has less work to do in extruding sodium. If less sodium is extruded, then less potassium may be replaced within the cell, if it is assumed that as in *Sepia* axons (Hodgkin and Keynes, 1954) the restoration of internal potassium is coupled with extrusion of sodium.

The action of chloroquine, mepacrine and pyrimethamine in arresting fibrillation may be

explained by effects which have recently been demonstrated. Armitage (1957) has shown that, when the isolated atria are exposed to the action of these substances, the contractions diminish in amplitude and frequency until they stop. They begin again when the external potassium concentration is lowered to one-half or one-quarter. This observation (also made with quinidine) suggests that these substances may make the membrane less permeable to potassium; the contractions may cease because the concentration gradient for potassium from inside to outside the cell is not then sufficient to allow exit of potassium. If, however, the external potassium is lowered, the potassium may once more travel out because the concentration gradient is raised. This explanation is supported by recent evidence that quinidine diminishes the uptake of K^+ by human red cells when cells kept at 0° C. for 3 to 5 days are then incubated at 37° C. (Kärki, Burn, and Burn, 1957).

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References

Armitage, A. K. (1957). *Brit. J. Pharmacol.*, **12**, 74.
—— Burn, J. H., and Gunning, A. J. (1957). *Circulation Research*, **5**, 98.
Burn, J. H., Vaughan Williams, E. M., and Walker, J. M. (1955). *J. Physiol.*, **128**, 277.
Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 90.
Dirken, M. N. J., Gevers, F., Heemstra, H., and Huizing, E. H. (1955). *Circulation Research*, **3**, 24.
Harris, E. J., and Hutter, O. F. (1956). *J. Physiol.*, **133**, 58P.
Hodgkin, A. L., and Keynes, R. D. (1954). *Sympos. Soc. Exper. Biol.*, **8**, 423.
Hoffman, B. F., and Suckling, E. E. (1953). *Amer. J. Physiol.*, **173**, 312.
Holland, W. C., Burn, J. H., and Schümann, H. J. (1957). *J. Pharmacol.*, in the press.
Kärki, N., Burn, G. P., and Burn, J. H. (1957). *Lancet*, **1**, 565.
McEwen, L. M. (1956). *J. Physiol.*, **131**, 678.
Saxby, O. B. (1956). Ibid., **133**, 4P.
Trautwein, K., Kuffler, S. W., and Edwards, C. (1956). *J. Gen. Physiol.*, **40**, 135.

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LEAD IONS AND SYNAPTIC TRANSMISSION IN THE SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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The perfused superior cervical ganglion of the cat has been used to study the effect of lead ions on synaptic transmission and on the relase of acetylcholine from preganglionic nerve endings. Lead ions, in concentrations of 5 to 40 μM./l., caused block of ganglionic transmission, and reduced the output of acetylcholine. Calcium ions (10 mM./l.) relieved the block produced by lead ions and restored the acetylcholine output. The presence of lead ions does not seem to change the sensitivity of ganglion cells to injected acetylcholine.

The present experiments were performed to test whether lead ions influence ganglionic transmission; a preliminary report has already appeared (Kostial, Vouk and Purec, 1954). Our investigations were limited to the influence of lead ions on the contractions of the nictitating membrane and on the output of acetylcholine (ACh.) in response to stimulation of pre- and post-ganglionic nerve fibres in perfused superior cervical ganglia of cats. The effect of increasing the concentration of calcium ions in the presence of lead ions was also studied.

METHODS

The cats were anaesthetized with chloralose and the superior cervical ganglion prepared for perfusion by the conventional method as modified by Perry (1953). The pre- and post-ganglionic trunks were stimulated at frequencies of 2 and 10 shocks/sec., and the contractions of the nictitating membrane recorded with an isotonic lever writing on a smoked paper. When ACh was to be collected, eserine sulphate (1:100,000) was added to the perfusion fluid and the postganglionic trunk was tied. Lead nitrate was added to Locke solution without altering the concentration of other components. In some experiments extra calcium chloride was added to the perfusion fluid; in others ACh (100 μg./ml.) was injected into the arterial cannula. On each change-over from one solution to another the dead space was flushed. Care was taken to avoid accumulation of fluid around the ganglion. ACh was assayed on the blood pressure of eviscerated cats anaesthetized with chloralose. The presence of lead ions in the perfusate did not appear to interfere with the assay of ACh.

RESULTS

Nictitating Membrane Contractions.—In order to test the influence of lead ions on ganglionic transmission, the concentration of lead in the perfusing fluid was gradually increased. Fig. 1 shows contractions of the nictitating membrane recorded during stimulation of the preganglionic nerve for 45 sec. at a frequency of 2 shocks/sec. In this experiment the superior cervical ganglion was perfused with Locke solution containing 2.4, 4.8 or 12.1 μM. of lead ions/l. Each perfusion with lead was followed by a perfusion with Locke

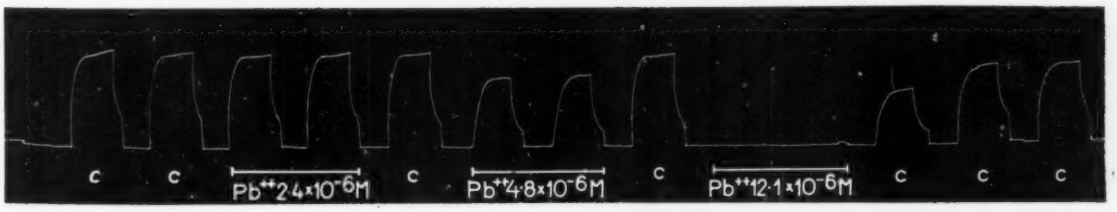

FIG. 1.—Contractions of the nictitating membrane of the cat in response to stimulation of the preganglionic trunk at 2/sec. for 45 sec. while perfusing the superior cervical ganglion with Locke solution (c) or with Locke solution containing increasing amounts of lead ions as shown.

solution. An effect of lead ions on the contractions of the nictitating membrane was first noticed at a concentration of 4.8 μM./l. ; complete block occurred at 12.1 μM./l.

In other experiments, the concentration of lead ions causing failure of nictitating membrane contractions ranged from 9.6 to 40 μM./l. In many instances the recovery of the nictitating membrane response was only partial on subsequent perfusion with Locke solution.

The results of an experiment in which pre- and post-ganglionic fibres were stimulated alternately are presented in Fig. 2. The superior cervical ganglion was perfused with Locke solution containing lead ions in increasing concentrations. The usual effect of lead ions was observed on preganglionic nerve stimulation. There was, however, no effect of lead ions on the contractions of the nictitating membrane during postganglionic stimulation. Conduction in postganglionic fibres did not seem to be affected by addition of lead ions to the perfusion fluid. After 19.3 μM./l. of lead ions the response to low frequency stimulation of the preganglionic nerve was scarcely perceptible, although the response to higher frequency of stimulation was only slightly reduced. This comparatively slight impairment of ganglionic transmission at the higher frequency of stimulation was always observed. A similar phenomenon has been observed when ganglionic transmission is blocked by magnesium ion (Hutter and Kostial, 1954).

FIG. 2.—Contractions of the nictitating membrane in response to stimulation of pre- or post-ganglionic fibres at 2/sec. for 45 sec. Lead ions suppressed the response of the nictitating membrane to preganglionic stimulation, leaving the response to postganglionic stimulation unaffected.

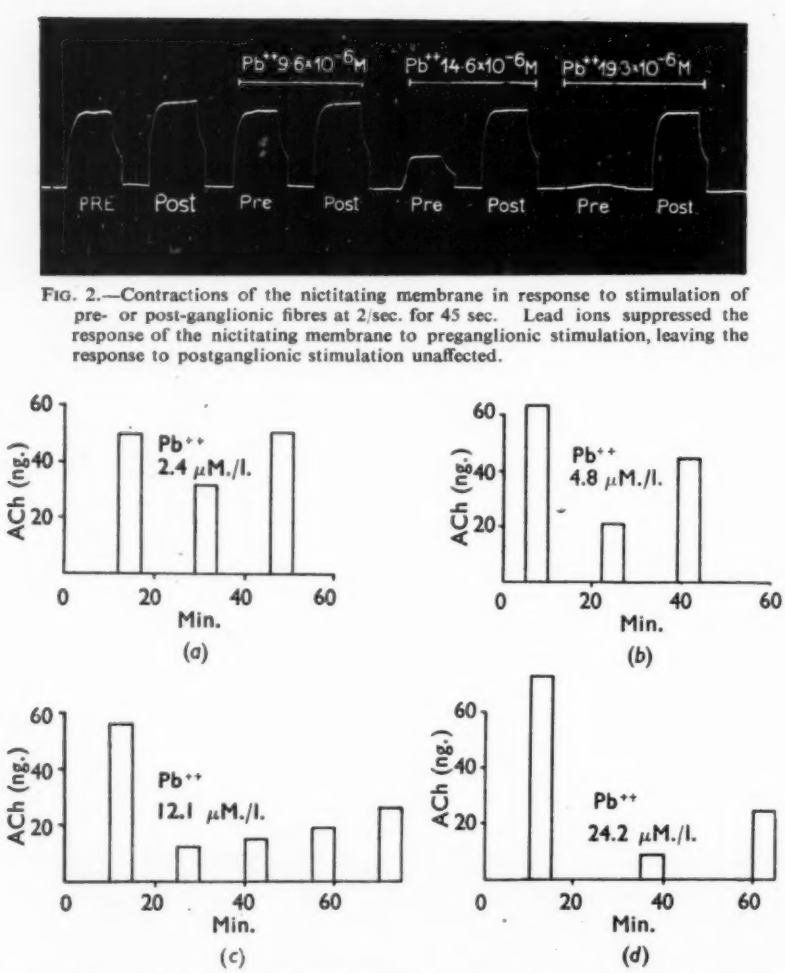

FIG. 3.—Ganglion perfused with Locke solution (eserine 10^{-5}). Each column represents the amount of ACh released during 5 min. of stimulation of the preganglionic nerve at 2/sec. In each section the ACh outputs before and after exposure to lead ions are shown. The ganglion was perfused with Locke solution containing lead ions in (*a*) 2.4 μM./l. for 17 min.; in (*b*) 4.8 μM./l. for 17 min.; in (*c*) 12.1 μM./l. for 30 min. (the second sample being taken 10 min. and the third 25 min. after exposure to lead), and (*d*) 24.2 μM./l. for 25 min.

Control experiments with sodium nitrate showed that nitrates in the concentrations used here had no influence on nictitating membrane contractions (Kostial *et al.*, 1954).

Effect of Lead Ions on Acetylcholine Output.—The influence of lead ions on the output of ACh is shown in Fig. 3 in which each column represents the amount of ACh collected over a period of 5 min. while stimulating the preganglionic nerve at a frequency of 2/sec. Fig. 3*a* gives the results of an experiment with 2.4 μM. of lead ions. The first sample was taken after perfusing the ganglion with normal Locke solution for 12 min. ; 2.4 μM./l. Pb^{++} was then added to the perfusion fluid and, after an exposure of 12 min., a 5 min. sample was taken. The third sample was collected 12 min. after changing over to Locke solution. A slight decrease in the output of ACh was noticeable. Fig. 3*b* shows an experiment with 4.8 μM./l. of Pb^{++} which caused the ACh output to fall to about 35% of its initial value, but recovery was only partial. With higher concentrations of lead ions the perfusate contained only very little ACh (Fig. 3*c* and *d*).

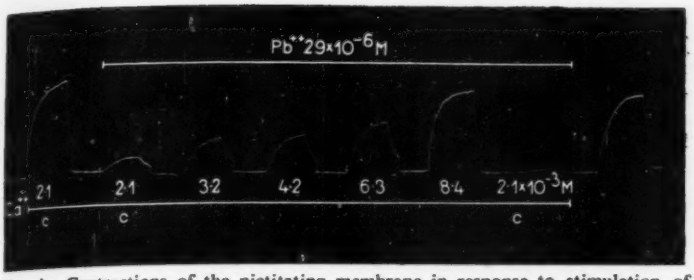

FIG. 4.—Contractions of the nictitating membrane in response to stimulation of preganglionic nerve. By increasing the concentration of calcium ions the block caused by the presence of lead ions was gradually relieved. C=Perfusion with Locke solution containing the normal concentration of calcium ions (2.1×10^{-3}M). The calcium ion concentration was raised to 3.2, 4.2, 6.3, and 8.4×10^{-3}M, and then returned to normal while perfusing the ganglion with Locke solution containing 29×10^{-6}M, lead ion concentration.

Antagonistic Effect of Calcium Ions.—The blocking action of lead ions is almost completely relieved by addition of calcium ions. When gradually increasing the concentration of calcium ions in the perfusion fluid containing 29 μM./l. of lead ions, complete recovery of nictitating membrane contractions to preganglionic stimulation was observed at a concentration of 8.4 mM. of calcium ions/l. (Fig. 4).

The antagonistic effect of calcium ions was also observed in experiments in which ACh output from the superior cervical ganglion was measured. Addition of 24.2 μM./l. of lead ions caused a pronounced reduction of the ACh output, with practically no recovery on returning to perfusion with Locke solution (Fig. 5, col. 3). By contrast, addition of five times the normal amount of calcium (10.5 mM.) to the perfusing solution containing 24.2 μM./l. of Pb^{++} restored the output of ACh to nearly its initial value. A similar restorative effect of calcium has been observed

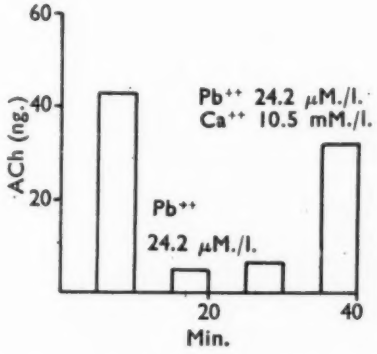

FIG. 5.—Ganglion perfused with Locke solution (eserine$\times10^{-5}$). Each column represents the amount of ACh released during 5 min. of stimulation of the preganglionic nerve at 2/sec. 24.2 μM./l. Pb^{++} was present during collection of sample 2. 24.2 μM./l. Pb^{++} and 10.5 mM./l. Ca^{++} was present during collection of sample 4.

when block is produced by magnesium ions (Hutter and Kostial, 1954).

Effect of Acetylcholine on Nictitating Membrane Contractions in Presence of Lead Ions.—Injection of ACh (10 μg. in 0.1 ml.) into the arterial cannula induced contractions of the nictitating membrane in a ganglion blocked to preganglionic stimulation by lead ions (Fig. 6). The sensitivity of ganglion cells to applied ACh did not appear to be altered by the presence of lead ions in the perfusion fluid.

DISCUSSION

Addition of lead ions to the perfusion fluid caused a partial or complete block of synaptic transmission indicated by failure of nictitating membrane contractions on preganglionic nerve stimulation. This failure in transmission was accompanied by a reduced output of ACh from

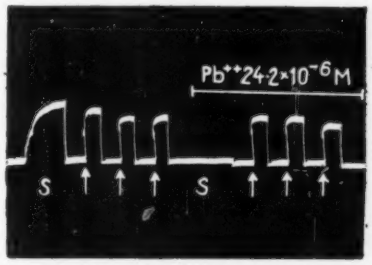

FIG. 6.—Responses of nictitating membrane to intra-arterial injections of ACh (10 μg. in 0.1 ml.) to the superior cervical ganglion at arrows. Three injections were made while perfusing with normal Locke solution, three after addition of 24.2×10^{-6}M. Pb^{++} to the Locke solution. S=Contractions in response to preganglionic nerve stimulation.

the superior cervical ganglion. The effects of lead ions could be reversed by increasing the calcium content of the perfusion fluid.

Several heavy metal ions (Cd^{++}, Cu^{++}, Ag^{+}, Hg^{++}, UO_2^{++}) cause an impairment of nerve conduction (Del Castillo and Hufschmidt, 1951). In our experiments, lead ions in concentrations causing a block of synaptic transmission did not seem to have an effect on nerve conduction. Contractions of the nictitating membrane occurred on stimulation of the postganglionic trunk in spite of the presence of lead ions. Furthermore, addition of ACh to the perfusion fluid containing enough lead to cause failure in synaptic transmission evoked contractions of the membrane.

The effects of lead ions on synaptic transmission were noticeable after 1 to 3 min. Mortensen and

Kellog (1944) found lead inside cells a few minutes after an intravenous injection. The temperature dependence of this reaction made these authors interpret the entrance of lead into the cell as an active chemical process. However, the speed of action of heavy metals points more to a cell surface action than to a diffusion process across the cell membrane. According to Davson (1951) heavy metals seem capable of blocking active groups in the cell membrane in accordance with their ability to inhibit enzyme catalysed reactions.

The antagonizing action of calcium ions might be explained by assuming a competitive action of lead and calcium ions for the same protein molecules, lead forming stronger protein complexes than calcium. According to Fatt (1954) calcium ions are essential for the functioning of an enzyme-like factor in the cell membrane, responsible for liberation of ACh from nerve endings. An excess of calcium ions might eliminate lead ions from their protein complexes and restore the normal activity of the enzyme-like factor in the membrane. It is very difficult to form any conclusive opinion on that subject, since very little is known about lead complexes with proteins (Cohn, Surgenor, Schmidt, Batchelor, Isliker and Alameri, 1953).

Lead ions inhibit resynthesis of phosphocreatine in the muscle (Steiman, 1939). Since we know that resynthesis of phosphocreatine also plays an essential role in the metabolism of ACh, we might assume that lead affects ACh synthesis. Del Castillo and Hufschmidt (1951) tried to explain the effect of heavy metals on nervous activity by their inhibiting action on SH-groups in the cell membrane. The SH-group is found to be a functional group of the co-enzyme A which is essential for acetylation of choline (Reisberg, 1954). Our results, however, cannot be explained by an effect of lead ions on ACh synthesis alone, since an impairment of transmission occurred before the store of ACh could be depleted (Perry, 1953).

Lead ions caused the same effect with and without addition of eserine to the perfusion fluid. The action of lead on cholinesterase (Frommel, Herschberg and Piquet, 1943, 1944), therefore, could not play an essential role in our experiments.

Our results seem to indicate the preganglionic nerve endings as the main site of action of lead ions. The influence of lead on nerve conduction, on ACh synthesis and on cholinesterase activity cannot be, however, entirely eliminated, but they do not seem to have an essential influence on our results.

We are indebted to Dr. O. F. Hutter and Mr. J. E. Pascoe for advice. We also wish to express our thanks to Miss Lj. Purec, from our laboratory, for her valuable assistance.

References

Cohn, E. J., Surgenor, D. M., Schmidt, K., Batchelor, W. H., Isliker, H. C., and Alameri, E. H. (1953). *Trans. Faraday Soc.*, **13**, 176.

Davson, H. (1951). *A Textbook of General Physiology*, p. 195. London: Churchill.

Del Castillo, J., and Hufschmidt, H. J. (1951). *Nature, Lond.*, **167**, 146.

Fatt, P. (1954). *Physiol. Rev.*, **34**, 674.

Frommel, E., Herschberg, A. D., and Piquet, J. (1943). *C.R. Soc. Physiol. Hist. Nat. Genève*, **60**, 97.

——— ——— ——— (1944). *Helv. physiol. acta*, **2**, 169.

Hutter, O. F., and Kostial, K. (1954). *J. Physiol.*, **124**, 234.

Kostial, K., Vouk, V. B., and Purec, Lj. (1954). *Arh. hig. rada.*, **5**, 251.

Mortensen, R. A., and Kellog, K. E. (1944). *J. cell. comp. Physiol.*, **23**, 11.

Perry, W. L. M. (1953). *J. Physiol.*, **119**, 439.

Reisberg, R. (1954). *Biochim. biophys. acta*, **14**, 442.

Steiman, S. E. (1939). *Amer. J. Physiol.*, **126**, 261.

Brit. J. Pharmacol. (1957), **12**, 223.

OXYTOCIC POLYPEPTIDES IN HUMAN URINE

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The work of Gomes on the polypeptide in human urine which causes contraction of the rat uterus has been extended. Concentrates were prepared by means of paper pulp columns similar to those used by Rocha e Silva and his colleagues for the purification of bradykinin, and evidence was obtained for the presence of two different active substances not only in human urine but also in a preparation of bradykinin. The effect of human urine on the rat uterus is probably due to bradykinin itself or a mixture of similar substances.

The substance responsible for the stimulating effect of crude urine and urinary extracts on the superfused rat uterus has been shown to have the properties of a polypeptide (Gomes, 1955). Evidence has been presented that the active principle closely resembled bradykinin and appeared to be related to that group of polypeptides which is represented by kallidin, bradykinin, substance U and substance Z (Gaddum, 1955; Werle and Erdös, 1954).

This report deals primarily with the purification of the substance. Some observations on bradykinin and its similarity with this substance are also included.

METHODS

Rat Uterus.—Uteri from virgin rats weighing 190 g. were superfused with de Jalon's solution (Gaddum, Peart and Vogt, 1949) by the technique described by Gaddum (1953).

Preparation of Alumina.—Aluminium oxide which is standardized (Savory and Moore) was found to absorb the active principle irreversibly. An acid washed alumina, as used by Pernow (1953) for the purification of substance P, was found to be an excellent medium for the purification of our principle. The aluminium oxide was washed twice with normal HCl for two hours. Then it was washed with distilled water until the *p*H of the washings was 6, dried at 90° C. and stored in a desiccator. The aluminium oxide columns were prepared as a slurry in 80% methanol which was allowed to settle by gravity. The material for purification was put on the column with 80% methanol and elution was effected with decreasing alcohol concentration. The solution was passed through the column by gravity or under slight pressure when necessary to maintain a flow of 1 ml./min. A 1.1×24 cm. column was used for 100 mg. of crude material, but a 1.1×8 cm. column is sufficient.

Paper Pulp Column.—The method of Andrade, Diniz and Rocha e Silva (1953), employing Whatman Cellulose Powder, which had proved successful in the purification of bradykinin, was used. The column was prepared by suspending the paper pulp in a 7% phenol solution and allowing the paper to settle by gravity until the desired height was reached. A 2.2×9 cm. column was found to be ideal for the purification of about 200 to 300 mg. of dry crude powder. The column was washed with additional 7% phenol, which removed a yellowish impurity from the paper pulp. The active principle was adsorbed from small volumes (5 to 10 ml.) of 7% phenol, which was also used as the developing solvent. Elution was effected with 0.4% acetic acid v/v and 0.1 N-HCl. The rate of elution was 4 ml./min. with the 7% phenol and 1 ml./min. with 0.4% acetic acid and 0.1 N-HCl. Of each 10 ml. fraction collected, 2 ml. was placed in a small test tube and lyophilized. The 2 ml. aliquots, after being lyophilized, were reconstituted with de Jalon's solution and assayed on the superfused rat uterus.

Paper Chromatography.—Ascending chromatography with Whatman No. 1 filter paper was used. The chromatograms were developed at room temperature (15 to 18° C.) for 18 to 24 hr. The solvents were acetone-N-butanol-HCl (10-4-2), and the organic phase of N-butanol, acetic acid and water (4-1-5) mixture.

The active substance was localized on the paper strips by biological assay on the superfused rat uterus. The simplest procedure was to cut the strip in 1 cm. portions, place in test tubes with 2 ml. of de Jalon's solution, allowing 5 to 10 min. for elution, and testing these solutions on the rat uterus.

Lyophilization.—Most of the purification operations were carried out in aqueous solutions. Because of the lability of the active principle, care had to be taken in reducing the volume of these solutions or in producing a dry powder by evaporation of the water solution. This

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was accomplished by lyophilization. No loss in activity occurred during the process. The dry powder was then directly reconstituted with de Jalon's solution and an assay performed.

When using the paper pulp column a few samples had phenol present. This was removed before lyophilization by ethyl ether extractions. The remaining ether in the solution was removed by freezing the sample and pouring off the small amount of ether.

The alcoholic solutions from the aluminium oxide columns were reduced in volume by vacuum distillation at 30 to 35° C. The remaining aqueous solution could then be frozen and lyophilized.

Preparation of a Standard Crude Powder.—The method of extraction was one described by Gomes (1955), and previously used by Clark, Winckler, Gollan and Fox (1954) for the extraction of hypertensin. The urine was acidified with HCl to *p*H 1.5, saturated with NaCl and extracted with an equal volume of N-butanol. The butanol was separated, filtered over anhydrous sodium sulphate and 5 volumes of ethyl ether (dry, distilled over sodium) added. This produced a flocculent precipitate which was allowed to settle overnight at room temperature. The ether was then decanted and the precipitate washed twice with fresh ether, collected on filter paper and stored in a desiccator. Approximately 1 g. of pinkish white powder was obtained from 1 l. of urine.

When this powder was prepared from samples of individual urines, it was found that the most potent preparation was three times more active than the weakest preparation obtained (Table I). This was probably due

TABLE I

VARIATION IN POWDERED EXTRACTS PREPARED FROM INDIVIDUAL URINES WHEN ASSAYED AGAINST A STANDARD PREPARATION FROM POOLED URINE

The unit is an arbitrary one (see text).

Preparation	Units/mg.
Standard	1·0
M.K.P.	2·4
E.J.W.	1·4
R.B.	0·95
D.S.	0·85
I.D.	1·0

to differences in salt concentration and not to any specific individual variation. In order to facilitate the evaluation of potency, a large sample of pooled urine (10 individuals) was used to prepare a standard preparation. This preparation was arbitrarily given the valuation of one unit of oxytocic activity per mg. of dry powder.

A partial purification of approximately 2 to 3-fold could be obtained by dissolving the crude dry powder described above in absolute methanol, filtering and adding 5 volumes of dry ethyl ether to the clear filtrate. A flocculent white precipitate resulted which was collected and stored in a desiccator. This preparation was not as stable as the crude dry powder, which was chosen as the standard preparation.

Chemicals.—The preparation of bradykinin used was kindly presented by M. Rocha e Silva. Its activity was given as 5 × Pool I (Rocha e Silva, 1955).

RESULTS

Hypertensin, which may be obtained by the extraction procedure used here (Clark *et al.*, 1954), has been further purified by using the cation exchanger IR-100H to remove salts. When this procedure was applied to our crude powder, the desalting was complete, but the recovery of pharmacological activity ranged from 20 to 40%.

Aluminium Oxide Columns.—Aluminium oxide, when prepared as described above, became a useful tool for the purification of the active principle. The results of two experiments are reported in Table II.

TABLE II

FRACTIONATION OF THE ACTIVE POLYPEPTIDE ON ALUMINIUM OXIDE COLUMNS

In Fractionation A the column measured 1·1 × 8·0 cm., and in Fractionation B a column 1·1 × 24 cm. was used.

	Fraction	Solvent: % Methyl Alcohol	Solvent: Vol. (ml.)	Dry Material (mg.)	Activity Units/mg.	Total Activity in Units
A	Original	95	50	100	1·0	100·0
	1	95	50	56	0·02	1·2
	2	70	50	4	4·5	18·0
	3	50	100	2	7·0	14·0
	4	Dist. water	35	25	0·28	7·0
					Sum	40·2
					Yield	40·2%
B	Original	80	80	75	1·0	75·0
	1	80	80	46·5	0·9	42·0
	2	70	80	3·0	7·9	23·8
	3	50	80	2·1	1·0	2·1
	4	50	120	4·0	0·88	3·5
	5	50	200	—	—	0·6
					Sum	72·0
					Yield	96%

The first experiment, using 95% methanol as the first solvent, showed a low recovery. Fraction 3 gave a 7-fold purification, but only a 14% recovery. The total recovery, when using high concentrations of methanol as the initial solvent, was only 40.2%. Starting with high concentrations of methanol offered the advantage of removing a great deal of weight with very little activity. The method using lower concentrations of methanol as the initial solvent necessitated careful cutting of fractions in order to achieve an increase in purification. Since the recovery was high, the remainder could always be rechromatographed for the fraction with the highest potency. Satisfactory results were obtained by the method using 80% methanol as initial solvent and 70% methanol as eluting solvent. This method consistently gave recoveries exceeding 85%. The method using absolute or 95% methanol as the initial solvent always gave recoveries of less than 50%. The longer column (1.1 × 24 cm.) was of some value in cutting the most active fractions, since, with a water-alcohol solution, the bands were very wide and tended to overlap on a smaller column (Table II).

Paper Pulp Column.—Andrade, Diniz and Rocha e Silva (1953) have used a paper pulp column successfully for the purification of bradykinin. Since the active substance in urine was similar to bradykinin in pharmacological activity (Gomes, 1955), the same method of purification was attempted.

The active principle is adsorbed on the paper pulp column from a 7% phenol solution and thus differs from hypertensin which is not adsorbed (Helmer, 1950). This is additional evidence that the activity is not due to hypertensin. Elution of an active fraction was effected with 0.4% acetic acid. Rocha e Silva used 0.2% acetic acid. Concentrations of 0.1%, 0.2%, 0.4%, 0.7% and 1% acetic acid were tried and 0.4% was found to be the most satisfactory. With this method, the recoveries of activity as assayed on the superfused rat uterus were from 30 to 60%.

The paper pulp column gave excellent results in purifying the crude powder preparation. With adsorption from the 7% phenol solution and subsequent development with this solvent, 90% of the weight of the original powder was found in the eluate. This powder was completely inactive on the superfused rat uterus in a concentration of 6.5 mg./ml. while the standard preparation was active in a concentration of 0.05 mg./ml. The original potency adsorbed on the column was equivalent to 250 mg. of the standard preparation.

The powder obtained from the acetic acid eluates of the paper pulp columns had activities ranging from 6 to 9 units/mg. where the starting material was 1 unit/mg.

In several purification experiments, when large volumes of the 0.4% acetic acid were passed through the column, a second output of activity was noticed. This was usually seen after 150 to 250 ml. of solution had been collected. Upon continued washing of the column (up to 650 ml.), this material continued to be detectable on the superfused rat uterus.

The active polypeptide preparation under these conditions thus showed the presence of two active substances. Various methods were tried to elute the remaining activity from the column and the most efficient eluting solvent found was 0.1 N-HCl. This brought down from 15 to 30% of the original activity. If combined with the acetic acid eluates the recoveries of the original activity ranged from 60 to 92% (Table III).

Provisionally, the substance eluted with acetic acid has been called Z_1 and that eluted with HCl, Z_2 (Fig. 1).

The technique used in the separation of Z_1 and Z_2 by the paper pulp column was as follows: About 400 mg. of the dry crude powder was dissolved in a small volume of distilled water, and a concentrated phenol solution added until the concentration of phenol was 7%. This solution was allowed to filter through the paper pulp column. About 150 to 250 ml. of the 7% phenol removed approximately 90% of the original weight. This was seen as a yellow band moving down the column. The eluate was a deep yellow colour. When the eluate became colourless, the 0.4% acetic acid was started. Usually 150 to 250 ml. was collected. Activity was detected in the first 10 ml. of the 0.4% acetic acid eluate, the peak being between

TABLE III

FRACTIONATION OF Z_1 AND Z_2 ON A PAPER PULP COLUMN

Fraction	Solvent	Vol. of Fraction (ml.)	Units/ml.	Total Units
Original	7% phenol	—	—	216
1	,,	250	0·0	0
2	0·4% acetic acid	50	1·2	60
3	,,	25	0·8	20
4	,,	25	0·4	10
5	,,	25	—	$\leq$ 1·5
6	,,	25	—	$\leq$ 1·5
7	,,	25	0·04	1
8	,,	25	0·2	5
9	,,	50	0·12	6
10	,,	50	0·1	5
11	0·1 N-HCl	75	0·4	30
12	,,	75	0·03	2
			Sum	140
			Yield	65%

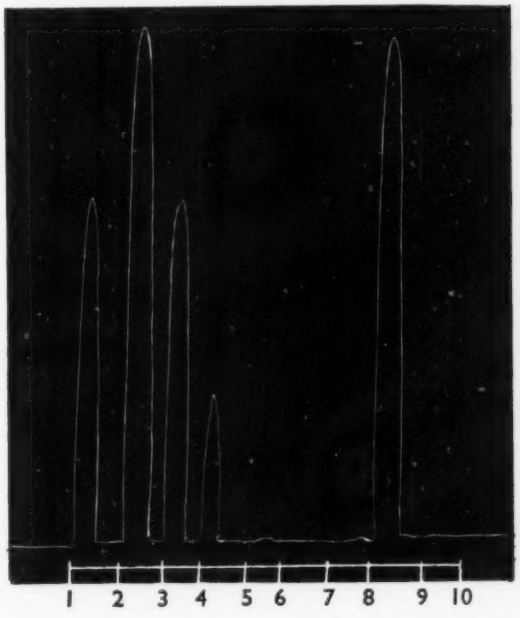

FIG. 1.—Rat uterus. Superfusion. Fractionation of Z_1 and Z_2 on the paper pulp column. Each response is due to a 1 : 5 dilution of an aliquot of 50 ml. fractions. Eluting fluids, 1 to 7 were 0.4% acetic acid, and 8 to 10 were 0.1 N-HCl.

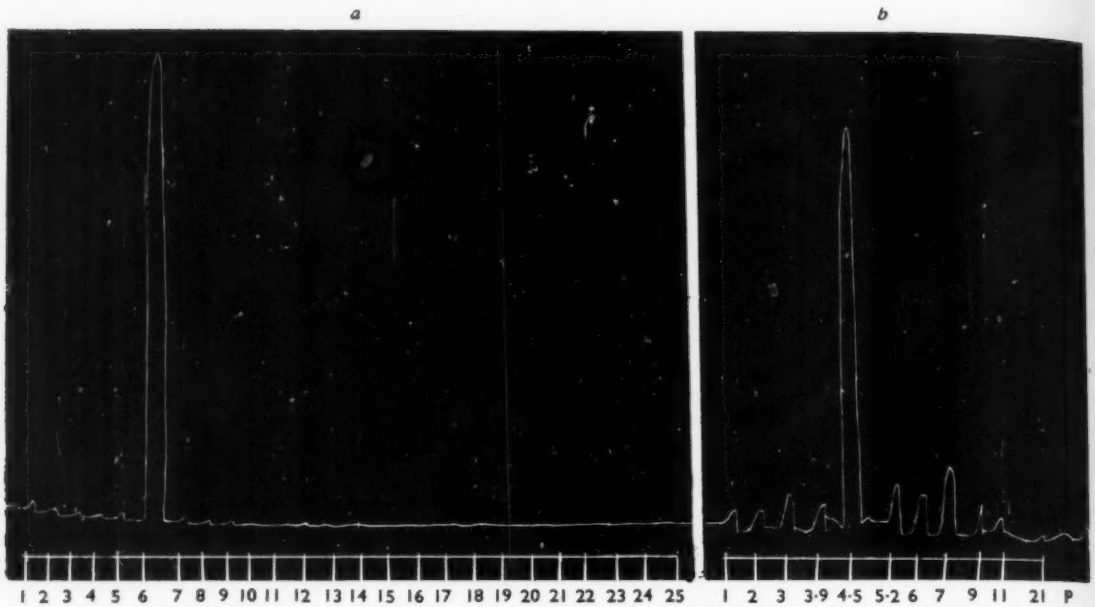

FIG. 2.—Rat uterus. Superfusion. Ascending paper chromatograms in butanol, acetic acid, and water. The numerals are the distance from starting line in cm. *a*, standard crude powder. R_F 0.22. *b*, mixture of standard crude powder and a preparation of bradykinin in equipotent amounts. R_F 0.2. P = paper only.

50 and 100 ml. The second eluting solution was 0.1 N-HCl. A pink band was then seen on the column. The first few ml. of the 0.1 N-HCl eluate contain almost all of the activity. It was best to collect the first few fractions in small volumes, as the later ones had less activity on a weight basis. The active fraction preceded the pink band. When the pink band was collected and dried, a red solid was obtained which had less activity than the preceding fraction. The first 50 ml. of eluate has 95 to 99% of Z_2, the next 50 ml. held the remainder and the red solid.

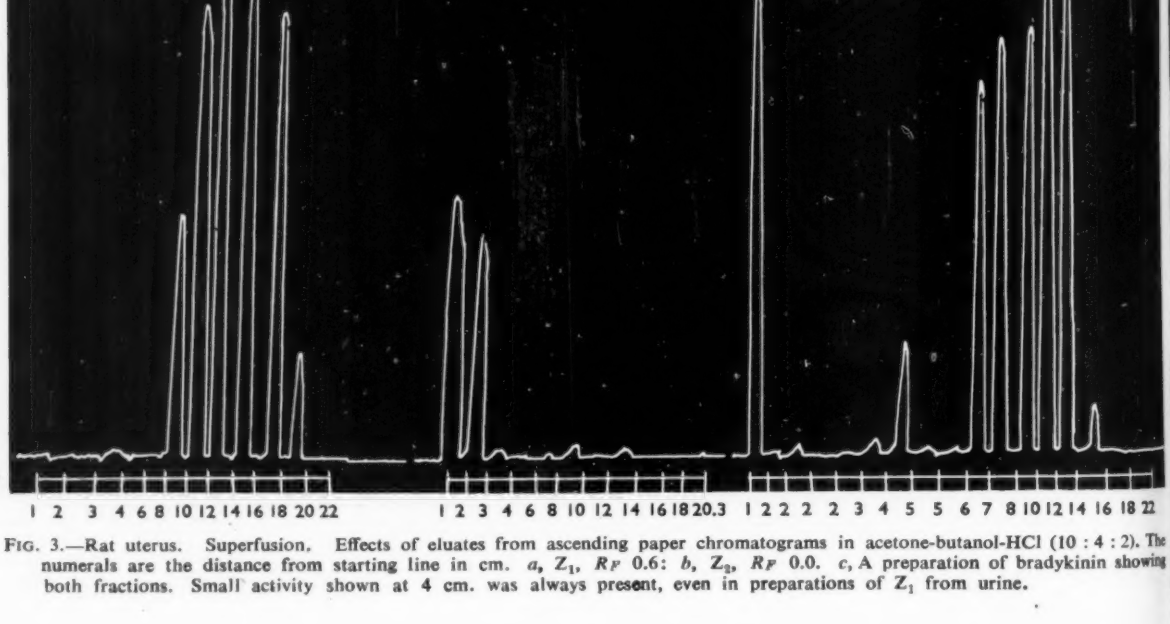

FIG. 3.—Rat uterus. Superfusion. Effects of eluates from ascending paper chromatograms in acetone-butanol-HCl (10 : 4 : 2). The numerals are the distance from starting line in cm. *a*, Z_1, R_F 0.6: *b*, Z_2, R_F 0.0. *c*, A preparation of bradykinin showing both fractions. Small activity shown at 4 cm. was always present, even in preparations of Z_1 from urine.

Combined Columns.—In an attempt to prepare a more purified preparation, the paper pulp and the aluminium oxide columns were used. Andrade *et al.* (1953) have shown that a combination of both columns was very successful in the purification of bradykinin. In these experiments, the crude powder was chromatographed on the paper pulp column and then chromatographed on the aluminium oxide column by the methods described above. Only Z_1 was purified in this way. The activity was 35 to 68 (35–44–68) times that of the original crude powder.

Paper Chromatography.—When an extract of the crude powder was chromatographed on paper using N-butanol-acetic-acid-water, the R_F of the activity was 0.2. It was lower when urine was used directly. Upon colour development with ninhydrin there are 5 spots on the chromatogram when the crude powder was chromatographed. In ultra-violet light, two spots were observed, one at the baseline and the other near the advancing front. The 5 spots with ninhydrin correspond to R_F values of 0, 0.1, 0.15, 0.2 and 0.35. The spot associated with the activity was pale violet in colour. If the strip was slightly overloaded with a larger amount of crude powder, a brownish spot was observed at R_F of 0.2. It seemed that the activity was always associated with this brownish spot which could be seen in daylight without any colour development.

The crude powder showed a spread of activity over the first 5 to 7 cm. of paper when chromatographed in the acetone, butanol and HCl system (Fig. 2). At times, the assay of the paper strip showed two peaks of activity, one at 0 cm. and another at 5 cm., but clearly defined results were never obtained. However, when Z_1 was chromatographed alone (Fig. 3*a*), a rather diffuse band was obtained with an R_F of 0.6. Z_2, in this system, gave an R_F of 0 (Fig. 3*b*).

A peculiarity noticed was that the R_F values of both Z_1 and Z_2 were 0 in the butanol, acetic acid and water (4 : 1 : 5) mixture, while the activity in the crude powder containing both Z_1 and Z_2 had an R_F of 0.2.

Comparison with Bradykinin

The similarity of this principle to bradykinin prompted a comparison of these two substances by paper chromatography. In the butanol-acetic-acid-water (4 : 1 : 5) system, the bradykinin had an R_F of 0.22, and the urine principle had an R_F of 0.20. When a mixed chromatogram was run (Fig. 2*b*), only a single peak of activity was noted.

When bradykinin was subjected to the chromatographic procedure for the separation of Z_1 and Z_2, it was found that it also gave two peaks, corresponding to Z_1 and Z_2. The preparation of bradykinin used in these experiments contained two active substances and the fraction corresponding to Z_2 in three different experiments contained 19, 21 and 25% of the total activity. The urinary extracts always contained a larger proportion of Z_2 than this, the average being 30% of the total activity. Bradykinin was also shown to contain two substances when chromatographed on paper in the acetone-butanol-HCl system (Fig. 3*c*). With this system, two active substances were found at R_F values corresponding roughly to those of the active substances in the urinary extract.

Discussion

The two active polypeptides found in urine seem to correspond to the two active polypeptides found in bradykinin. Gomes (1955) has previously shown that the active polypeptide found in urine resembled bradykinin when subjected to parallel assays on the rat uterus, guinea-pig ileum and hen rectal caecum. In our experiments, bradykinin was shown to have the same chromatographic properties as the urinary substance, and, furthermore, the preparation of bradykinin used contained two active polypeptides similar to those found in the purification of the urinary substance. The possibility exists that this bradykinin-like substance is a naturally occurring compound which is manufactured in the human body and partly destroyed by enzymes in blood and kidney cortex, and partly excreted in the urine (Hamberg and Rocha e Silva, 1954; Rocha e Silva, 1955).

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References

Andrade, S. O., Diniz, C. R., and Rocha e Silva, M. (1953). *Arch. int. Pharmacodyn.*, **95**, 100.
Clark, L. C., Winckler, C., Gollan, F., and Fox, R. P. (1954). *J. biol. Chem.*, **206**, 717.
Gaddum, J. H. (1953). *Brit. J. Pharmacol.*, **8**, 321.
—— (1955). In *Polypeptides which Stimulate Plain Muscle*. Edinburgh : Livingstone.
—— Peart, W. S., and Vogt, M. (1949). *J. Physiol.*, **108**, 467.
Gomes, F. P. (1955). *Brit. J. Pharmacol.*, **10**, 200.
Hamberg, U., and Rocha e Silva, M. (1954). *Acta physiol. scand.*, **30**, 215.
Helmer, M. O. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 642.
Pernow, B. (1953). *Acta physiol. scand.*, **29**, Suppl. 105.
Rocha e Silva, M. (1955). In *Polypeptides which Stimulate Plain Muscle* (J. H. Gaddum, ed.), p. 51. Edinburgh : Livingstone.
Werle, E., and Erdös, E. G. (1954). *Arch. exp. Path. Pharmak.*, **223**, 234.

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ANTICONVULSANT COMPOUNDS AND 5-HYDROXY-TRYPTAMINE IN RAT BRAIN

BY

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In rats, a series of anticonvulsant compounds have been shown to cause a significant elevation of brain 5-hydroxytryptamine (5-HT) levels in comparison with control values. This increase in 5-HT only occurred in brain tissue and was not observed in spleen, upper small intestine or blood. Elevation of brain levels of 5-HT by iproniazid (Marsilid) or 5-hydroxytryptophan failed to give protection against the convulsant or lethal action of lept zol (75 mg./kg.).

The effect of phenytoin (diphenylhydantoin, Dilantin) in preventing the response of the pituitary-adrenal system to various standard procedures, known to cause a discharge of the adrenocorticotrophic hormone (ACTH) and a resultant fall in adrenal ascorbic acid, has been studied both for a direct action of the drug upon the adrenal gland itself and a possible alteration in some central mechanism (Bonnycastle and Bradley, 1956, 1957). As it appeared fairly clear that, initially at least, the interference with the response is central in origin since the adrenal cortex remains normal in its responsiveness to exogenous trophic hormone, it was of interest to examine the levels of one or other of the suggested transmitting substances in the brain. In a preliminary report (Bonnycastle, Paasonen and Giarman, 1956) the 5-hydroxytryptamine (5-HT) content of the brains of rats treated with phenytoin was shown to be elevated approximately twofold over that of untreated control animals. The present paper deals with the brain levels of 5-HT in rats treated with other anti-epileptic drugs in use. Unlike phenytoin, none of these substances has any inhibitory effect upon the pituitary-adrenal system.

METHODS

Male rats of the Sprague-Dawley strain weighing 100 to 300 g. were used in these experiments. The compounds listed in Table I were examined. While the dosage varied considerably, an attempt was made to use doses which have been reported to be anticonvulsant in the rat against one or other convulsant procedure.

Since many of the substances used are insoluble in any suitable media, some problems of uniform administration arose. After some preliminary experiments, the most satisfactory method was to weigh out the individual doses and suspend them as microcrystalline preparations for intraperitoneal injection. No attempts were made to determine the minimal effective dose or the duration of effect. The original dosage regimen was based on the previous study (Bonnycastle and Bradley, 1956) and usually consisted of four doses given over two days, the animals being

TABLE I

EFFECT OF SOME ANTICONVULSANT COMPOUNDS UPON THE CONCENTRATION OF 5-HYDROXYTRYPTAMINE IN RAT BRAIN

The numerals in brackets in col. (3) indicate number of doses given. The difference between the values for the treated and control rats was in each instance, highly significant ($P < 0.001$). The numerals in brackets in col. (6) give the standard error.

Drug		Individual Dose (mg./kg.) (3)	Time after Last Dose (hr.) (4)	No. of Rats (5)	Conc. in Brain of 5-HT (ng./g.) (6)
Chemical Name (1)	Name or Synonym (2)				
Control				20	341 (±10·5)
5, 5 diphenyl hydantoin	Phenytoin Dilantin	100(16)	12	5	703 (±85)
5 ethyl, 3 methyl, 5 phenylhydantoin	Methion Mesantoin	200 (4)	12	3	607 (±46)
3, 5, 5 trimethyl-2, 4 oxazolidinedione	Troxidone Tridione	150 (4)	12	9	481 (±13·7)
3, 5 dimethyl 5 ethyloxazolidine, 2, 4, dione	Parametha-dione Paradione	200 (4)	12	3	501 (±10·0)
5, 5 diphenyloxazolidine, 2, 4 dione	Epidon	400 (4)	12	2	508 (±12·5)
N-methyl phenyl succimide	Phensux-amide Milontin	450 (4)	12	4	574 (±27)
Phenylacetyl urea	Phenace-mide Phenurone	500 (4)	12	6	561 (±20)
5 phenyl, 7 ethyl hexahydropyrimidine, 4, 6 dione	Primidone Mysoline	500 (4)	12	4	54[illegible] (±7·8)
5 ethyl-5 phenyl barbituric acid	Phenobarbi-tone	125 (1)	2·5	5	532 (±34·5)
5 phenyl-5-ethyl-3 methyl barbituric acid	Prominal Gemonil Mebaral	125 (1)	2·5	4	524 (±28·0)
Sodium bromide		1,000 (5)	4	4	531 (±38)

killed by decapitation 12 hr. after the last dose. In some instances, as noted in Table I and in the text, there has been a departure from this design.

The brains (without cerebella) were quickly removed and homogenized with an equal volume of isotonic saline in a Potter-Elvehjem type homogenizer. Extracts were then made of the total homogenates by the method of Amin, Crawford, and Gaddum (1954). When it was desired to determine the 5-HT content of tissues other than brain, these were first minced with scissors in saline, then homogenized and extracted in the same manner. The 5-HT content of the various extracts was determined by bioassay, using the heart of the mollusc *Venus mercenaria* suspended in sea-water in a 4 ml. over-flow type bath. The sea-water contained benzoquinonium (Mytolon) in a concentration of 6 mg./l., to prevent interference by choline esters in the extracts. This method of assay, introduced by Twarog and Page (1953) and used extensively by Gaddum and Paasonen (1955), has in our hands yielded reliable and reproducible estimates of 5-HT in biological tissues in the range of 1 to 10 ng.

The significance of the difference between levels found in the treated animals and the control figure is determined by Student's *t* test.

Results

In Table I it is seen that treatment of rats with various anticonvulsant drugs led to a significant increase in the brain levels of 5-HT. This increase was measured 12 hr. after the last dose, except in the case of sodium bromide and phenobarbitone and its methyl derivative. In view of the rather slow displacement of chloride by bromide a single daily dose was given for five days and the animals examined 4 hr. after the last dose. No change in brain 5-HT was observed 12 hr. after a single dose of phenobarbitone or the methyl derivative (325 to 380 ng./g. and 330 to 350 ng./g. respectively). When the 5-HT content was determined in rat brains 2.5 hr. after administration of a single dose of either of these barbiturates, it was found to be increased. These results suggested the possibility that only one dose was necessary with other drugs. It was found that methion (Mesantoin), paramethadione (Paradione), phensuxamide (Milontin), phenacemide and primidone all produced elevated values of brain 5-HT 12 hr. after a single dose of the size indicated in Table I.

The question was raised whether this action of the anticonvulsant drugs was limited solely to brain tissue, or whether their administration would also affect the 5-HT levels of other tissues. Table II presents the results of single experiments in which estimates were made of the effect of five of these compounds on venous blood (vena cava), small intestine and spleen. The results clearly indicate that no alteration occurred in the 5-HT content of tissues other than brain.

Table II

5-HYDROXYTRYPTAMINE CONTENT OF SOME BODY TISSUES OF RATS TREATED WITH ANTICONVULSANT AGENTS COMPARED WITH CONTROLS

Each set of results is derived from a single rat. The numerals in brackets indicate number of doses.

Treatment	Dose (mg./kg.)	Time after Last Dose (hr.)	Brain (ng./kg.)	Spleen (ng./kg.)	Small Intestine (ng./kg.)	Venal Cava Blood (ng./kg.)
None ..			340	8,100	4,800	780
,, ..			360	4,600	6,600	400
,, ..			450	3,700	3,800	450
,, ..			420	3,700	3,100	430
Phenytoin ..	100 (7)	12	580	3,200	2,900	400
Phenacamide	450 (1)	12	710	4,600	2,400	420
Phensuxamide	400 (1)	12	740	6,400	2,700	430
Parametha-dione	400 (1)	2·5	520	4,900	2,900	400
Phenobarbi-tone	125 (1)	2·5	710	5,500	4,900	520

In attempting to explain the elevation of levels of brain 5-HT by the anticonvulsant drugs, it seemed of some interest to determine whether the action might be due to inhibition of monoamine oxidase. With rat liver homogenates and tyramine as a substrate, it was not possible to demonstrate manometrically any such inhibition by phenytoin. Similarly phenytoin in amounts of 0.1 to 0.2 mg./ml. failed to influence the destruction by rat liver homogenates of added 5-HT. In addition, preliminary experiments by differential centrifuging have indicated no striking alteration of the normal pattern of distribution of 5-HT within rat brain cells after treatment with phenytoin or phenacemide.

The finding that anticonvulsant compounds led to an increase in brain 5-HT made it of interest to measure what effect, if any, this elevation would have upon the convulsant activity of leptazol (75 mg./kg.). Some rats were treated with iproniazid (100 mg./kg. of base) 11 hr., and others with 5-hydroxytryptophan (100 mg./kg.) 30 min. before administration of the above dose of leptazol. The results are given in Table III, from which it is clear that the elevated brain levels of 5-HT (which

Table III

CONVULSANT ACTION OF LEPTAZOL IN RATS PREVIOUSLY TREATED WITH IPRONIAZID (MARSILID) OR WITH 5-HYDROXYTRYPTOPHAN

	No. Showing Convulsions	Mortality	Survival Time (Min.)	
			Mean	Range
Control group ..	7/10	6/10	16	(5 to 30)
Iproniazid-treated group	7/10	5/10	56	(10 to 120)
5-hydroxytryptophan-treated group ..	6/10	6/10	16	(5 to 45)

is known from previous work to be about 800 ng./g. in the case of iproniazid, and 925 ng./g. in the case of 5-hydroxytryptophan) afforded little protection against the convulsant action of leptazol. The survival times of the animals that died in the treated groups showed some differences from the control group. Two of the deaths in the iproniazid group occurred 2 hr. after the administration of leptazol, while all those in the 5-hydroxytryptophan group occurred during the first 45 min.

Studies with meprobamate (Miltown), which has been said to exert a beneficial effect in some forms of epilepsy, such as petit mal (Perlstein, 1956), gave no indication of an alteration in the brain levels of 5-HT in the rat when examined about 2 hr. after administration. The brain 5-HT concentrations in treated rats were 322, 435, 334, and 329 ng./g. compared with a control of 341 ng./g. On the other hand, pentobarbitone in anaesthetic doses of 50 mg./kg. resulted in an elevation of brain levels of 5-HT after 45 min. to 524, 592, 565, and 540 ng./g. compared with a control value of 341 ng./g. Treatment with hexobarbitone (Evipan) in an initial anaesthetic dose of 100 mg./kg. with two supplements resulted in levels of 518 and 485 ng./kg. in the two cases which were examined after 45 min. These findings might have been expected in view of the results obtained with phenobarbitone and its methyl derivative, but nevertheless they raised the question of a possible association between the elevation of brain 5-HT and the action of the central depressant drugs in general. It was clear from the experiments carried out so far that the time at which these effects were seen differed with different drugs, and some of the apparent negative findings which have been observed may well be due to the fact that the examination was made at the wrong time.

Discussion

Very few compounds have been discovered which alter the brain levels of 5-HT. It is known that reserpine and the related substances rescinnamine and desoxyreserpine cause all tissue stores of 5-HT to be depleted. Only two types of substance have been reported to cause an elevation of tissue levels of 5-HT. Inhibition of monoamine-oxidase apparently permits some accumulation of 5-HT to take place, and the inhibitor most commonly used for this purpose is iproniazid (Marsilid). The other compound which has been shown to raise the tissue levels of 5-HT is its immediate precursor, 5-hydroxytryptophan The findings obtained in the present study are of some interest, for here there are a number of pharmacologically related compounds, all of which lead to an increase in the brain level of 5-HT in the rat. From the results obtained it appears probable that this increase is not merely the result of repeated dosage of the drugs, but can occur following a single dose of any one of the drugs, provided that it is sought at the appropriate time. No attempt was made to ascertain the minimal effective dose in any case, but it is very probable that they are lower than the figures reported in Table I. In only one instance was any examination made of the time course of the effect, and this was with phenobarbitone with which no appreciable alteration from control levels was detectable at 1.5 to 2 hr. (390 to 400 ng./g.) and 12 hr. (325 to 380 ng./g.) after treatment, but values as high as 600 ng./g. were found 2.5 hr. after treatment.

The existence of a relationship between an increase in brain 5-HT and the anticonvulsant action of these drugs is certainly open to question. It has been observed in rats that such convulsing procedures as electro-shock, or the administration of leptazol, picrotoxin and CO_2, do not change the brain levels of 5-HT at least under the conditions of the experiments (Paasonen and Giarman, unpublished observations; Bonnycastle, unpublished observations; Giarman and Golden, unpublished observations). The results in Table III make it clear that elevation of brain 5-HT obtained either by monoamine-oxidase inhibition or by supplying the precursor afforded no protection against the convulsant action of leptazol (75 mg./kg.). Although there was a somewhat longer survival time in the iproniazid-treated group, the ultimate mortality rate was similar in the three groups of animals. It will be of interest to determine the effect of some of these anticonvulsant compounds upon the brain levels of 5-HT in other species, particularly those in which it is possible to dissect discrete neural structures. It will also be of interest to determine the time relationships of the changes in brain levels of 5-HT and the anticonvulsant activity of some of these drugs.

The results given in Table II serve a dual purpose. They show that the effects produced by the anticonvulsant drugs are restricted to the cerebral tissues, but also they serve as internal standards for the experiments. It is to be expected that some of the drug or drug metabolites would be distributed in tissues other than brain, and the fact that the values for 5-HT in the other tissues examined were within the limits of the control figures answers, in part at least, the question as

to what effect these substances might have upon the system used for assay. The control levels for spleen and upper small intestine reported here are higher than available figures obtained by others (Erspamer, 1954 ; Paasonen and Giarman, unpublished observations), and this is probably due to the method of preparing the extracts. In one of the studies (Paasonen and Giarman), the tissues were only minced with scissors before extraction with acetone, while in the present study tissues were first minced with scissors and then homogenized before extraction. It is probable that this procedure would allow a more complete extraction, and thus account for the higher values reported here for these tissues.

The mechanism by which these anticonvulsant compounds bring about the observed increase in brain 5-HT is not known, but preliminary studies suggest that it is not the result of monoamine-oxidase inhibition.

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References

Amin, A. H., Crawford, T. B. B., and Gaddum, J. H. (1954). *J. Physiol.*, **126**, 596.

Bonnycastle, D. D., and Bradley, A. (1956). *Fed. Proc.*, **15**, 403.

—— —— (1957). In the press.

—— Paasonen, M. K., and Giarman, N. J. (1956). *Nature*, **178**, 991.

Erspamer, V. (1954). *Rendiconti Scientifici Farmitalia.*, **1**, 5.

Gaddum, J. H., and Paasonen, M. K. (1955). *Brit. J. Pharmacol.*, **10**, 474.

Perlstein, M. A. (1956). *J. Amer. med. Ass.*, **161**, 1040.

Twarog, B. M., and Page, I. H. (1953). *Amer. J. Physiol.*, **175**, 157.

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THE ACTION OF ERGOTAMINE ON THE INTRACRANIAL VENOUS PRESSURE AND ON THE CEREBRAL VENOUS OUTFLOW OF THE DOG

BY

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The effect of ergotamine and dihydroergotamine on the cerebral circulation was studied in the dog, anaesthetized with chloralose, by recording the intracranial venous pressure and the venous outflow from the superior cerebral vein. Under optimal experimental conditions, ergotamine (5 to 10 μg./kg.) and dihydroergotamine (100 μg./kg.) gave a marked and long-lasting cerebral vasoconstriction accompanied by a slight hypertension. The cerebral vasoconstriction provoked by ergotamine may be very small and was sometimes absent when the cerebral blood-flow was low. This vasoconstrictor effect is more pronounced the higher the initial intracranial venous pressure and hence the cerebral blood-flow. After the induction of a cerebral vasodilatation by 48/80 or strychnine, the vasoconstrictor action of ergotamine was more pronounced. The effect was not observed when CO_2 was employed to modify the intracranial venous pressure. Simultaneous registration of the cerebral, nasal cavity, and kidney vascular responses demonstrated the relative specificity of the action of ergotamine on the cerebral vessels. The small doses of ergotamine used may weaken or abolish the vasoconstrictor action of adrenaline on cerebral vessels. The modification of the responses of the cerebral circulation which such factors as anaesthesia, respiratory acidosis, and operative trauma can produce have been confirmed and emphasized. The results support the view that the vasoconstrictor action of ergotamine on the cerebral vessels might account for the therapeutic value of this drug in migraine.

Although the efficacy of ergotamine in the treatment of migraine has been well known for a long time (Thomson, 1894 ; Maier, 1926 ; Tzanck, 1928 ; Lennox, 1934), the mechanism of its action is still a subject for discussion. The suggestion, based upon clinical observations, that ergotamine exerts its therapeutic effect through a vasoconstrictor action on the cranial vessels (Lennox and von Storch, 1936 ; Graham and Wolff, 1938 ; Wolff, 1948) has hitherto received little experimental confirmation. Systematic investigations carried out in animals and men have very rarely demonstrated a clear cerebral vasoconstrictor action of ergotamine even when large doses were administered.

The direct observation of pial vessels (Ley and de la Fontaine-Verwey, 1929 ; Pool and Nason, 1935), tissue thermometry (Lubsen, 1940 ; Schmidt, 1934), perfusion methods (Heymans and Regniers, 1927), blood flow measurements in the internal carotid artery (Schneider and Schneider, 1934b ; Dumke and Schmidt, 1943), plethysmographic techniques, registration of cerebrospinal fluid pressure (Koopmans, 1939 ; Lennox, Gibbs and Gibbs, 1935 ; Cannavà and Musmeci, 1940) and the nitrous oxide method (Abreu, Liddle, Handley, and Elliot, 1947 ; Abreu, Liddle, Burks, Simon, Sutherland, and Gordan, 1948) applied to various animal species and to man (Lennox *et al.*, 1935 ; Abreu *et al.*, 1948) have never shown a clear-cut vasoconstrictor action of ergotamine. Such effects sometimes followed substantial doses (0.05 to 1.0 mg./kg.) or intracarotid injection (Dumke and Schmidt, 1943 ; Schneider and Schneider, 1934b ; Koopmans, 1939 ; Ley and de la Fontaine-Verwey, 1929 ; Pool and Nason, 1935). Heymans and Regniers (1927), Schmidt (1934), Lennox *et al.* (1935), Cannavà and Musmeci (1940), Lubsen (1940), and Abreu *et al.* (1947, 1948) reported that ergotamine caused a rise of the cerebral blood-flow which according to Lennox *et al.* (1935), depended on the arterial hypertensive action of the alkaloid.

Previous observations made in this laboratory on the registration of the cerebral venous pressure through the various branches of the external jugular vein in the dog anaesthetized with chloralose have confirmed that this method enables the cerebral vasomotor effect of various drugs to be studied (Bovet, Gatti and Virno, 1955a and b; Virno, Gertner, and Bovet, 1956; Bovet, Virno, Gatti, and Carpi, 1957). The object of the present study was to obtain further information on the vasomotor effect of ergotamine on the cerebral circulation. In order to obtain a closer parallel between clinical and laboratory observations, care was taken to observe the effect of minimal doses. The experiments were carried out both under normal conditions and in animals previously treated with cerebral vasodilator agents.

Methods

In the dog anaesthetized with chloralose (120 mg./kg., i.v.) the peripheral venous pressure was registered by means of a polyvinyl catheter introduced into the external jugular vein and manipulated by way of the internal maxillary and the superior cerebral veins until it reached as near as possible to the orifice of the temporal foramen. Nasal cavity volume was recorded according to the method of François-Frank (1894), modified in some details (Bovet *et al.*, 1957), and the renal volume was registered plethysmographically by means of Roy's oncometer. In some experiments, records of systemic venous pressures were registered by means of a catheter introduced through the external jugular vein into the superior vena cava.

For all these recordings water-manometers were employed, and the changes in the height of the column of water were recorded photographically according to the method of Baldes and Corbeile (1929) modified by Condorelli (1941).

In other experiments, the peripheral jugular pressure and the outflow from the superior cerebral vein were simultaneously registered. A second polyvinyl catheter was introduced into the opposite external jugular vein and manipulated, as previously described, towards the orifice of the temporal foramen. This catheter was used for recording the venous outflow and its free orifice was fixed on the same level as the external jugular vein. It was so arranged that the drops of blood short-circuited a pair of contacts connected through an electronic relay with a Fleisch's summator which automatically returned to zero every 15 sec. The blood was then recirculated into the external jugular vein at the same rate as the outflow. Blood clotting was prevented by the use of 10 mg./kg. of X50 (Geigy Ltd.). The exact position of the catheters was always verified by a dissection at the end of the experiments. Femoral blood pressure was recorded with a Ludwig's mercury-manometer.

Respiratory acidosis was induced by two different methods. In the first experimental group, the dog was allowed to breathe gas mixtures containing 10 to 30% CO_2 in air. The second group of animals were given gallamine in a sufficient quantity to paralyse the skeletal musculature and were given artificial respiration. Hypercapnia was obtained here by reducing the minute volume to approximately 1/5 of the normal value. Air was in this case substituted by oxygen to avoid anoxemia and the arterial blood *p*H was repeatedly measured by means of a glass-electrode.

All drugs were administered into the saphenous vein.

Results

Effect of Ergotamine on the Cerebral Vessels and the other Vascular Areas under Normal Conditions.—When 5 to 10 μg./kg. of ergotamine was administered to an animal it was observed that, besides the well-known rise in arterial pressure (10 to 20 mm. Hg), a significant fall in the cephalic venous pressure occurred; the initial level of 15 to 20 cm. H_2O fell to 8 to 12 cm. H_2O, remaining at this pressure for many hours (Fig. 1). Under these experimental conditions, after ergotamine, the peripheral jugular pressure did not return to its original level even after a period of 4 hr. This effect is less pronounced if the peripheral pressure is already low before the ergotamine administration (Fig. 6).

In 10 animals, the average fall of cephalic venous pressure provoked by 5 to 10 μg./kg. of ergotamine was 3.5 cm. H_2O (1.5 to 7.0 cm. H_2O). Compared with the action of adrenaline (1 to 3 μg./kg.) this effect of ergotamine was more intense in 3, equal in 4 and weaker in 1 of eight experiments undertaken. Compared with the long-lasting action of ergotamine, the effect of adrenaline on the peripheral jugular pressure lasted no more than 2 to 4 min.

Repeated injections of the alkaloid do not give rise to a further lowering of the cephalic venous pressure. Very often a slight increase in the peripheral jugular pressure together with a marked rise of arterial pressure was observed, especially if larger doses of the drug were injected. This was only partially related to the low level of the peripheral jugular pressure after the first administration of the drug.

The outflow from the superior cerebral vein was decreased by ergotamine and this reduction ran strictly parallel to the fall in the peripheral jugular pressure. For example in one experiment it was observed that a fall from 15 to 11.5 cm. H_2O in the cephalic venous pressure corresponded to a 30% decrease in the venous outflow of the opposite side.

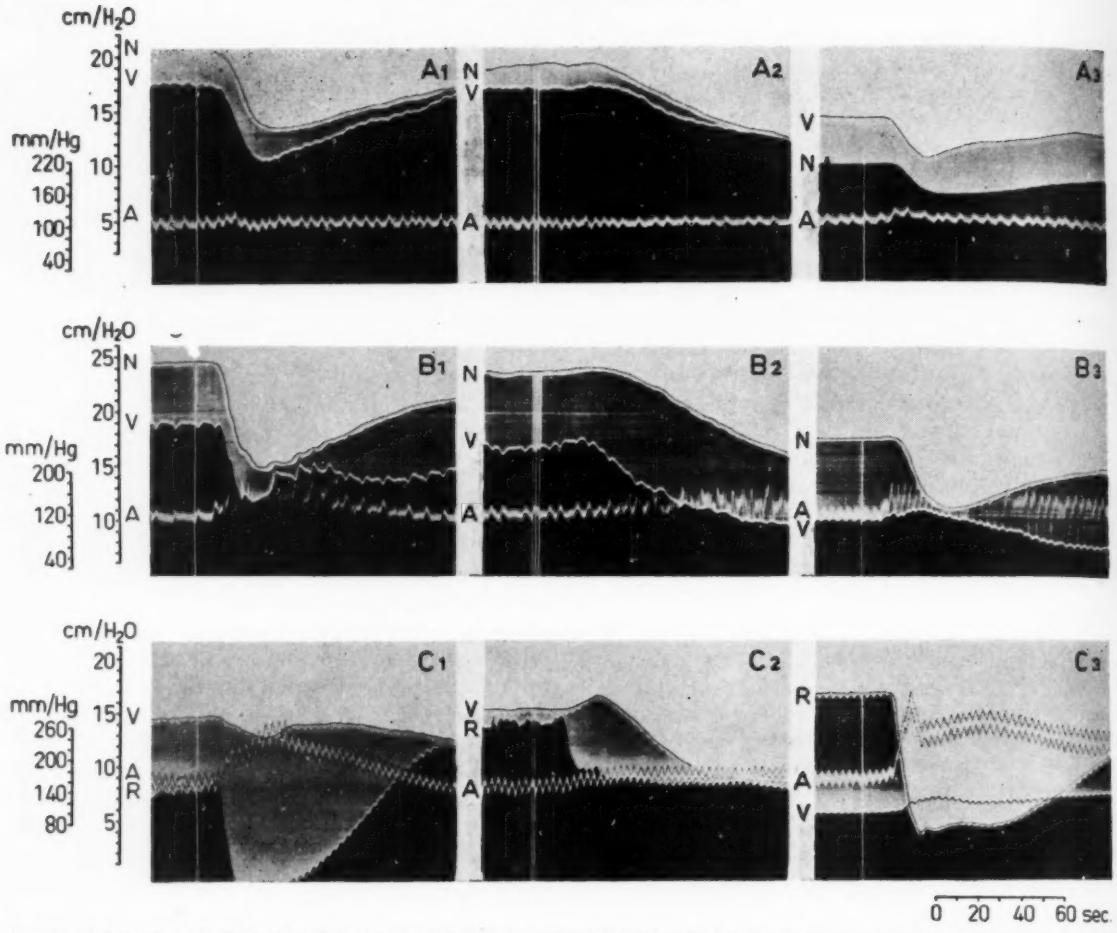

FIG. 1.—Effect of small doses of ergotamine on the cephalic venous pressure, on the nasal and the renal plethysmogram and its antagonism to the action of adrenaline. In all tracings A = pressure in the femoral artery (mm. Hg); V = peripheral pressure in the internal maxillary vein (cm. H_2O); N = nasal cavity plethysmogram; R = renal plethysmogram. A_1, A_2, and A_3, male dog 14 kg. (21/6/56), chloralose anaesthesia; A_1, adrenaline 0.1 μg./kg.; A_2, ergotamine 5.0 μg./kg.; A_3, adrenaline 0.1 μg./kg. B_1, B_2, and B_3, male dog 18 kg. (13/6/56), chloralose anaesthesia; B_1, adrenaline 1 μg./kg.; B_2, ergotamine 10 μg./kg.; B_3, adrenaline 1 μg./kg. C_1, C_2, and C_3, male dog 16 kg. (24/7/56), chloralose anaesthesia; C_1, adrenaline 1 μg./kg.; C_2, ergotamine 10 μg./kg.; C_3, adrenaline 1 μg./kg.

The doses of ergotamine which lowered the cranial venous pressure also caused a constriction of the nasal vessels which corresponded to an increase in the total volume within the nasal cavity. Consequently a fall of the water column in the manometer connected to the closed nasal cavity was observed (Fig. 1, A_1, A_2, A_3, B_1, B_2, and B_3). In a series of 15 experiments it was observed that this vasoconstrictor effect on nasal vessels lasted in 7 cases more than 2 hr., whereas in the other 8 it disappeared 30 to 45 min. after the ergotamine injection. The intensity of the nasal vasoconstriction provoked by 5 to 10 μg./kg. of ergotamine was similar to that produced by 1 μg./kg. of adrenaline in 5 of 8 animals and less in the other 3. Ergotamine in the usual dose of 5 to 10 μg./kg. caused a slight and transient fall in the kidney volume which was always less than that provoked by 1 μg./kg. of adrenaline (Fig. 1, C_1, C_2, and C_3). The alkaloid had only a slight effect on the systemic venous pressure; a rise of 1 to 2 cm. H_2O after administration of 5 to 10 μg./kg. i.v. ergotamine was observed.

Influence of Ergotamine on the Cerebral Vascular Action of Adrenaline. — With the small doses used (5 to 10 μg./kg.), ergotamine did not clearly modify the arterial hypertension provoked by 0.1 to 3 μg./kg. of adrenaline (Fig. 1). Sometimes this action was slightly potentiated as previously observed by Hartman (1915), Woods, Nelson, and Nelson (1932), Herwick, Linegar and Koppanyi (1939), and Von Euler and Schmiterlöw (1944). The renal vasoconstrictor response to adrenaline was unaltered after ergotamine, and the

nasal vasoconstriction only partially reduced (Fig. 1). On the other hand the action of adrenaline on the cephalic venous pressure was clearly modified and closely related to the level of the peripheral jugular pressure at the moment of the injection of the hormone. In all cases where the injection of ergotamine produced a fall in the venous pressure which was equal to or more pronounced than that provoked by adrenaline, the further injection of adrenaline did not produce an additional fall in the cephalic venous pressure (Fig. 1, B_1, B_2, B_3, C_1, C_2, and C_3). However, if the jugular venous pressure after the injection of ergotamine did not fall to the level produced by adrenaline in the normal animal then a further injection of adrenaline might produce a further fall of the cephalic venous pressure (Fig. 1, A_1, A_2, and A_3). After doses of ergotamine (0.5 to 1 mg./kg.) large enough to produce anti-adrenaline actions, the typical adrenaline reversal reaction on the arterial blood pressure was observed; in this case the venous pressure followed passively the fall of the arterial pressure provoked by adrenaline.

Effects of Dihydroergotamine on the Cerebral Vessels and on the Cerebral Vascular Action of Adrenaline.—Dihydroergotamine in doses of 100 μg./kg. has a similar action on the peripheral jugular pressure and on the cerebral venous outflow as 5 to 10 μg./kg. of ergotamine. After such doses of dihydroergotamine, the action of adrenaline (1 to 3 μg./kg.) on the cephalic venous pressure was the more reduced the greater the fall in the peripheral jugular pressure provoked by the alkaloid.

Interaction Between the Vasoconstrictor Effect of Ergotamine and the Action of Some Cerebral Vasodilators.—From the results described, it was clear that ergotamine caused a fall in the peripheral jugular pressure which was more pronounced the higher the cephalic venous pressure before the administration of the alkaloid. Hence the action of ergotamine after the induction of a cerebral vasodilatation which produced a marked rise in cephalic venous pressure was studied. The histamine liberator 48/80, strychnine, and gas mixtures with high content of CO_2 were used, and the effect of ergotamine on the action of these vasodilator agents was investigated.

Compound 48/80 caused a marked and long-lasting rise in the peripheral jugular pressure. Under these conditions 5 to 10 μg./kg. of ergotamine significantly reduced the cephalic venous pressure to a level which was lower than that prior to the administration of the histamine-liberator. Consequently the injection of 48/80 before ergotamine gave rise to a more substantial effect of the

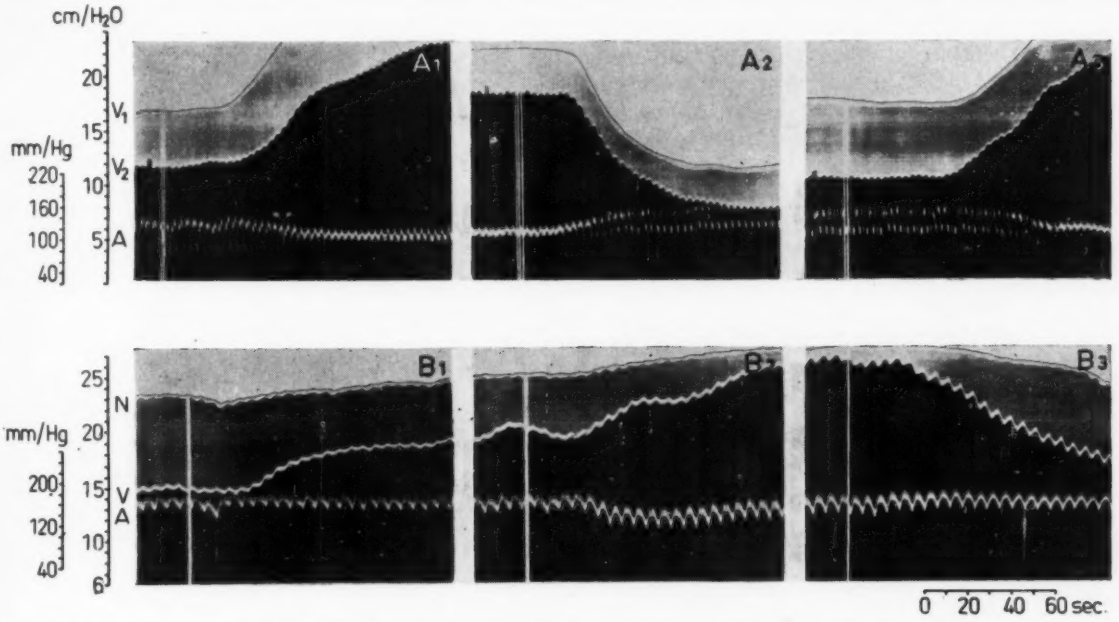

FIG. 2.—Interaction between 48/80 and ergotamine on the cephalic venous pressure and the nasal vessels. A_1, A_2, and A_3, male dog 14 kg. (1/7/55), chloralose anaesthesia. A=pressure in the femoral artery (mm. Hg); V_1=peripheral pressure in the external maxillary vein (cm. H_2O); V_2=peripheral pressure in the internal maxillary vein (cm. H_2O). A_1, 48/80 25 μg./kg.; A_2, ergotamine 10 μg./kg.; A_3, 48/80 50 μg./kg. B_1, B_2, and B_3, male dog 11 kg. (3/7/56), chloralose anaesthesia. A=pressure in the femoral artery (mm. Hg); V=peripheral pressure in the internal maxillary vein (cm. H_2O); N=nasal cavity plethysmogram. B_1, 48/80 25 μg./kg.; B_2, 48/80 25 μg./kg.; B_3, ergotamine 10 μg./kg.

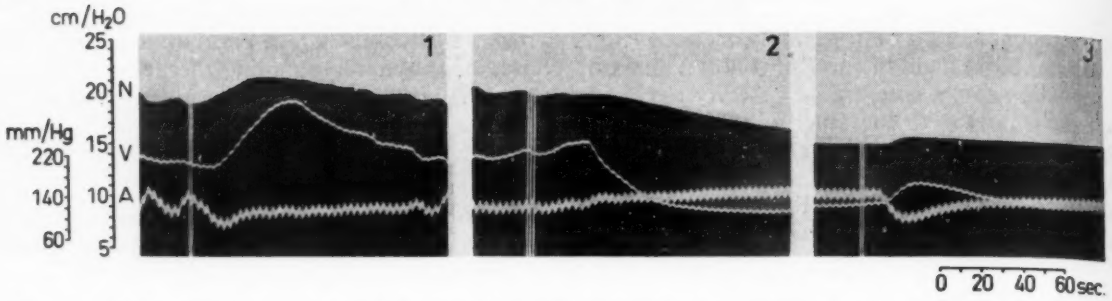

FIG. 3.—Action of histamine on the cephalic venous pressure, the nasal plethysmogram and its antagonism by ergotamine. Recordings as in Fig. 1. Female dog 14 kg. (25/5/56), chloralose anaesthesia. 1, histamine 1 μg./kg.; 2, ergotamine 10 μg./kg.; 3, histamine 1 μg./kg. i.v.

alkaloid (Fig. 2). In four experiments, the average fall of the peripheral jugular pressure caused by the alkaloid was 11.5 cm. H_2O (10.5 to 14.5 cm. H_2O). On the nasal vessels, the long-lasting vasodilator action of 48/80 is also evident without any marked change in the vasoconstrictor action of ergotamine being observed (Fig. 2, B_1, B_2, and B_3).

As repeated injections of 48/80 do not provoke constant vascular effects, it was difficult to obtain precise information about the influence of ergotamine on the action of the histamine-liberator; in only three such experiments a typical action of 48/80 in large doses on the peripheral jugular pressure was observed (Fig. 2, A_1, A_2, and A_3).

As previous experiments (Virno *et al.*, 1956) showed that histamine administered in doses of 0.5 to 1 μg./kg. gave a marked but brief rise of the cephalic venous pressure, a comparison was made of the effect of histamine itself before and after ergotamine injections. It was found that ergotamine (5 to 10 μg./kg.) moderately reduced the vasodilator action of histamine in 9 of 12 experiments (Fig. 3).

In anaesthetized dogs, which had received a dose of gallamine (2 to 4 mg./kg.) and were respired artificially, an intravenous injection of 100 to 500 μg./kg. of strychnine gave a slow but long-lasting rise in the cephalic venous pressure and an increase in the outflow of the superior cerebral vein. This effect was often coupled with some rise in arterial pressure (20 to 50 mm. Hg). Under these conditions, ergotamine caused a reduction in the peripheral jugular pressure and in the cerebral venous outflow to a level lower than that observed before strychnine (Fig. 4A). In nine experiments, the average fall of cephalic venous pressure caused by ergotamine (5 to 10 μg./kg.) was 7.0 cm. H_2O (3.5 to 12.0 cm. H_2O). Dihydroergotamine had the same effect (Fig. 4, B_1, B_2, and B_3). Strychnine did not give uniform results on the nasal cavity vessels: thus in one dog there was vasodilatation, in another vasoconstriction, whereas in

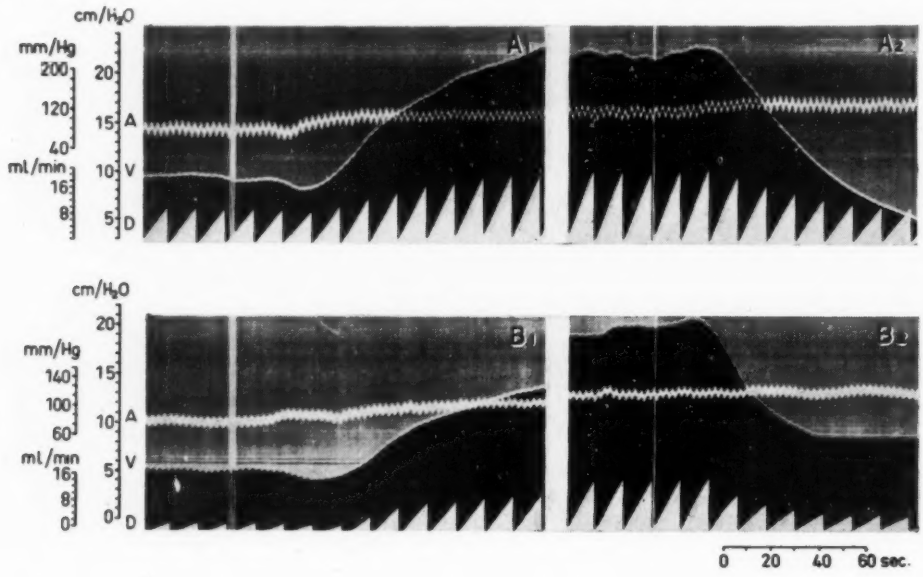

FIG. 4.—Influence of strychnine on the action of ergotamine and dihydroergotamine on the cephalic venous pressure and on the outflow from the superior cerebral vein. A and V as in Fig. 1. D=outflow from the left superior cerebral vein (ml./min.). A_1 and A_2, male dog 16 kg. (16/10/56), chloralose anaesthesia. A_1, strychnine 0.5 mg./kg.; A_2, ergotamine 10.0 μg./kg. B_1 and B_2, male dog 8 kg. (8/10/56), chloralose anaesthesia. B_1, strychnine 0.5 mg./kg.; B_2, dihydroergotamine 0.1 mg./kg. Both animals were maintained under artificial respiration after gallamine (4 mg./kg.).

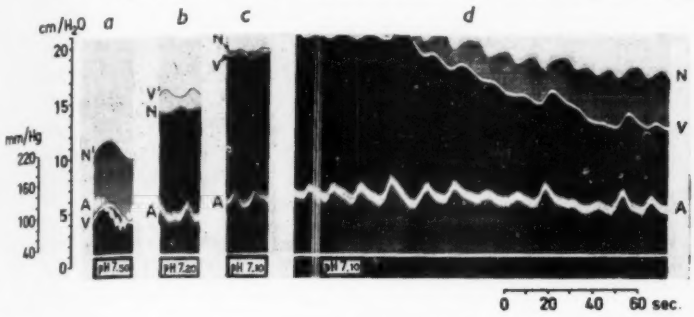

FIG. 5.—Influence of respiratory acidosis on the action of ergotamine on the cephalic venous pressure and on the nasal plethysmogram. Records as Fig. 1. The *p*H was determined from samples of the femoral arterial blood. Male dog 19 kg. (13/3/56) under chloralose anaesthesia after gallamine (1 mg./kg.). *a*, *p*H 7.5, artificial respiration with 2.5 l./min. O_2. *b*, *p*H 7.2, after 15 min. of artificial respiration with 0.45 l./min. O_2. *c*, *p*H 7.1, after 30 min. of artificial respiration with 0.45 l./min. O_2. *d*, *p*H 7.1, after 35 min. of artificial respiration with 0.45 l./min. O_2. At the three vertical lines ergotamine (10 μg./kg.) was injected.

three no change was observed. The vasoconstrictor effect of ergotamine on the nasal vessels in these experiments was the same as under normal conditions.

Because of the long-lasting action of strychnine, it was difficult to compare in the same dog the action of the drug before and after ergotamine. However, in 14 of the dogs, the administration of 500 μg./kg. of strychnine was clearly devoid of any effect on the arterial and on the peripheral jugular pressure 20 to 30 min. after the injection of 10 μg./kg. of ergotamine. Only in two other dogs did strychnine, administered 2 hr. after ergotamine, give a moderate vascular effect. It was also observed that 48/80 and histamine were still active in dogs which, after the administration of ergotamine, did not respond to strychnine.

Respiratory acidosis was induced in one series of experiments with dogs, which had received gallamine and which were maintained by artificial respiration, by a reduction of the minute volume to 1/5th of the normal value, oxygen being substituted for air, and in another series of dogs under normal respiration by the administration of gas mixtures containing 10 to 30% CO_2 in air. Hypercapnia caused a marked rise in the peripheral jugular pressure which could not be completely abolished by ergotamine (Fig. 5).

In six experiments, the fall in the cephalic venous pressure produced by ergotamine was, on an average, 4.5 cm. H_2O (3.5 to 7.0 cm. H_2O) and generally lasted up to 15 min. The vasodilator action of CO_2 on the nasal vessels was evident in 4 of the 6 experiments and was often preceded by a brief vasoconstrictor reaction ; vasoconstriction after ergotamine was always weaker in these animals than under normal conditions (Fig. 5) and in 2 cases it was practically absent.

In some experiments the effect of ergotamine on the vascular reactions induced by respiratory acidosis was studied. Hypercapnia was obtained by administration of gas mixtures containing 40 to 50% CO_2 in O_2 during 10 or 20 respiratory cycles. Under these conditions there was a marked rise in the cerebral venous pressure which lasted 9 to 10 min., and a biphasic reaction of the nasal vessels which first constricted and then dilated with slight and inconsistent changes in arterial blood pressure.

Ergotamine in the usual dose of 5 to 10 μg./kg. weakened the effect of hypercapnia on the cephalic venous pressure and even more markedly on the nasal vessels (Fig. 6). It was also observed that, after ergotamine, hypercapnia caused a fall in the arterial pressure as previously described by Heymans and Bouckaert (1933). This fall in systemic pressure was transient and could not have interfered significantly with the reaction of the peripheral jugular pressure (Fig. 6).

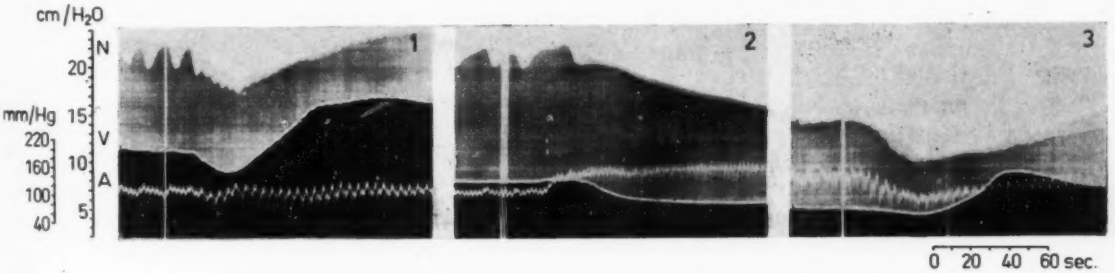

FIG. 6.—Effect of short-lasting hypercapnia on the cephalic venous pressure and nasal plethysmogram, and its antagonism by ergotamine. Records as in Fig. 1. Female dog 19 kg. (15/3/56), chloralose anaesthesia. 1, administration of a gas mixture containing 50% CO_2 in O_2 during 10 respirations. 2, ergotamine (10 μg./kg.). 3, administration of the same gas mixture during 10 respirations.

Discussion

The experiments reported here confirm that changes in the cerebral venous pressure as measured by means of a catheter introduced into the peripheral portion of the external jugular vein may be considered of significance in the study of the cerebral vascular reactions produced by the administration of various drugs. The fact that the arterial and peripheral jugular pressures may vary independently and often in opposite directions (Bovet *et al.*, 1955a and b ; Virno *et al.*, 1956 ; Bovet *et al.*, 1957) was confirmed and interpreted as demonstrating a direct effect of drugs on the peripheral resistance of the brain vessels. Under various experimental conditions, quite different reactions were noted on the cerebral, extracerebral and visceral circulation.

The conclusions drawn from these observations on venous pressure receive further support by experiments carried out on cerebral venous outflow ; generally a fall in the venous pressure of 80% corresponded approximately to a 50% diminution in the outflow.

Ergotamine injected at a dose of 5 to 10 μg./kg. in the dog anaesthetized with chloralose exerted a distinct cerebral vasoconstrictor action, which was clearly demonstrated by the fall in the peripheral jugular pressure and the decrease of the blood flow in the cerebral vein.

The main reason for the conflicting results obtained by various authors on the cerebral vascular action of ergotamine may be due to the high susceptibility of the cerebral vessels to various noxious and toxic agents. It has previously been shown that, under some experimental conditions such as barbiturate anaesthesia, hypercapnia, and minor traumatic shock, adrenaline and noradrenaline do not have a vasoconstrictor action (Bovet *et al.*, 1957). The appearance of this refractoriness to the vasoconstrictor action of the drugs is usually coupled with a progressive and marked fall in the peripheral jugular pressure. Similar factors manifested themselves in the response to ergotamine. It is clear that the cerebral vasoconstrictor effect of ergotamine was markedly affected by the initial level of the peripheral jugular pressure. It may be totally absent in cases of refractoriness of the cerebral vessels in which the venous outflow was initially low. Thus some of the negative results reported in the literature may be attributed to the trauma induced by the technique employed.

The significance of the initial level of the peripheral jugular pressure at which cerebral vasoconstrictor action of ergotamine appeared is emphasized by the fact that this action of the alkaloid becomes much more evident after drugs such as 48/80 and strychnine which caused a marked rise in the cephalic venous pressure. This action of 48/80 on the venous pressure has previously been described (Virno *et al.*, 1956) and appears to be related to the histamine-liberator effect.

The action of strychnine on the cerebral circulation was due to an increase in the systemic arterial pressure and an active vasodilatation of the cerebral vessels. The fact that ergotamine provoked a strong cerebral vasoconstrictor action in animals which had received strychnine is particularly significant, as the effect of strychnine on the cerebral circulation is probably related to the analeptic action of strychnine. Thus the responses of the cerebral vessels in the anaesthetized dog receiving strychnine resemble more closely those in the normal rather than those in the anaesthetized animal.

The experiments on the effect of CO_2 and acidosis upon cerebral venous pressure demonstrate the rôle played by this factor in brain circulation (Bouckaert and Jourdan, 1949 ; Lennox and Gibbs, 1932 ; Schmidt, 1928). In the markedly hypercapnic animal, the vasoconstrictor effect of ergotamine appeared generally to be weak and transient, a fact which corroborates the previous observations on the lack of a cerebral vasoconstrictor action of adrenaline under respiratory acidosis (Bovet *et al.*, 1957).

The influence of ergotamine on the cerebral vascular action of other drugs may be considered next. The abolition of the action of adrenaline on the peripheral jugular pressure by such small doses of ergotamine as 5 to 10 μg./kg. cannot be ascribed to the anti-adrenaline action of the alkaloid, which only appears after administration of much larger quantities. Clearly, the suppression of the cerebral vascular action of adrenaline is related to the well-known vasoconstrictor action of the alkaloid. After ergotamine, the peripheral jugular pressure passively follows the changes in arterial pressure provoked by adrenaline, similar results having been obtained under various experimental conditions by Forbes, Finley, and Nason (1933), Schneider and Schneider (1934a) and Taeschler, Cerletti, and Rothlin (1952).

The influence of small doses of ergotamine on the cerebral vascular action of the various vasodilator agents here studied, namely 40/80, histamine, and CO_2, must be considered as a functional antagonism depending on the vasoconstrictor effect of the alkaloid. Experiments, undertaken

to compare the relative activity of ergotamine and adrenaline on cerebral nasal and renal vessels, showed that, whereas small doses of adrenaline generally provoked comparable effects on these three vascular sites, the action of similar doses of ergotamine was slight on the renal plethysmogram, moderate and often transient on the nasal vessels, and more pronounced and of longer duration on the cerebral vessels. This series of experiments clearly demonstrated a relative specificity of action of ergotamine on the cerebral vessels. The dose used experimentally (5 to 10 μg./kg.) corresponds to that commonly injected clinically (0.5 mg. or 7 μg./kg.) and these experiments on the dog confirm the observations in man (Lennox and von Storch, 1936; Von Storch, 1937; Graham and Wolff, 1938; Wolff, 1948) and support the cerebral vasoconstrictor explanation of the value of ergotamine in the relief of migraine.

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References

Abreu, B. E., Liddle, G. W., Handley, C. A., and Elliot, H. W. (1947). *Fed. Proc.*, **6**, 304.
—— —— Burks, A. L., Simon, A., Sutherland, V., and Gordan, G. S. (1948). Ibid., **7**, 201.
Baldes, E. J., and Corbeile, C. (1929). *Proc. Soc. exp. Biol., N.Y.*, **26**, 711.
Bouckaert, J. J., and Jourdan, F. (1949). *J. Physiol. Path. gen.*, **41**, 69A.
Bovet, D., Gatti, G. L., and Virno, M. (1955a). *Helv. physiol. acta*, **13**, C6.
—— —— —— (1955b). *R.C. Acad. Lincei*, serie VIII, **19**, 67.
—— Virno, M., Gatti, G. L., and Carpi, A. (1957). *Arch. int. Pharmacodyn.*, in the press.
Cannavà, A., and Musmeci, S. (1940). *Arch. Farmacol. sper.*, **69**, 73.
Condorelli, L. (1941). *Fisiopatologia del circolo venoso.* Catania: A.P.E.
Dumke, P. R., and Schmidt, C. F. (1943). *Amer. J. Physiol.*, **138**, 421.
Euler, U. S. v., and Schmiterlöw, C. G. (1944). *Acta physiol. scand.*, **8**, 122.
Forbes, S. K., Finley, K. H., and Nason, G. I. (1933). *Arch. Neurol. Psychiat., Chicago*, **30**, 957.
François-Frank, C. A. (1894). *Titres et travaux scientifiques*, 123.
Graham, J. R., and Wolff, H. G. (1938). *Arch. Neurol. Psychiat., Chicago*, **39**, 737.
Hartman, F. A. (1915). *Amer. J. Physiol.*, **38**, 438.
Herwick, R. P., Linegar, C. R., and Koppanyi, T. (1939). *J. Pharmacol.*, **65**, 185.
Heymans, C., and Bouckaert, J. J. (1933). *C.R. Soc. Biol., Paris*, **112**, 717.
—— and Regniers, P. (1927). *Arch. int. Pharmacodyn.*, **33**, 236.
Koopmans, S. (1939). *Arch. néerl. Physiol.*, **24**, 250.
Lennox, W. G. (1934). *New Engl. J. Med.*, **210**, 1061.
—— and Gibbs, E. L. (1932). *J. clin. Invest.*, **11**, 1155.
—— —— and Gibbs, F. A. (1935). *J. Pharmacol.*, **53**, 113.
—— and von Storch, T. J. C. (1936). *Amer. J. med. Sci.*, **192**, 57.
Ley, J., and de la Fontaine-Verwey, B. C. (1929). *C.R. Soc. Biol., Paris*, **101**, 478.
Lubsen, N. (1940). *Arch. néerl. Physiol.*, **25**, 361.
Maier, H. W. (1926). *Rev. Neurol.*, **33**, 1104.
Pool, J. L., and Nason, G. I. (1935). *Arch. Neuro'. Psychiat., Chicago*, **33**, 226.
Schmidt, C. F. (1928). *Amer. J. Physiol.*, **84**, 202.
—— (1934). Ibid., **110**, 137.
Schneider, M., and Schneider, D. (1934a). *Arch. exp. Path. Pharmak.*, **175**, 606.
—— —— (1934b). Ibid., **175**, 640.
Taeschler, M., Cerletti, A., and Rothlin, E. (1952). *Helv. physiol. Acta*, **10**, 120.
Thomson, W. H. (1894). *J. Nervous Mental Disease*, **19**, 124.
Tzanck, M. A. (1928). *Bull. Soc. méd. Hôp., Paris*, **44**, 1057.
Virno, M., Gertner, S. B., and Bovet, D. (1956). *J. Pharmacol.*, **118**, 63.
Von Storch, T. J. C. (1937). *New Engl. J. Med.*, **217**, 247.
Wolff, H. G. (1948). *Headache and Other Head Pain.* New York: Oxford University Press.
Woods, G. G., Nelson, V. E., and Nelson, E. E. (1932). *J. Pharmacol.*, **45**, 403.

Brit. J. Pharmacol. (1957), **12**, 240.

THE EFFECT OF CORTISONE ON THE MULTIPLICATION OF *M. TUBERCULOSIS* IN NORMAL AND IMMUNE MICE

BY

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The multiplication of *M. tuberculosis, var. bovis* inoculated into the cornea of mice was studied by staining the whole cornea at various stages after inoculation. Four groups of animals were studied: untreated animals, animals treated with cortisone, animals previously immunized with the same bovine strain, and immunized animals treated with cortisone.

In the untreated immunized group little or no multiplication occurred. In the other three groups multiplication did occur and was about the same for the first week after inoculation. After this stage, cortisone-treated animals, whether previously immunized or not, showed increased multiplication and massive cord formation, as compared with untreated animals in which little further multiplication was seen. The cortisone treatment had thus completely suppressed immunity. The significance of these results is discussed.

When tubercle bacilli of a virulent strain are injected into the cornea of a mouse, progressive multiplication of the organisms occurs until at a certain stage a macroscopic lesion appears which spreads to involve most of the cornea. The infection, however, does not spread systemically, and the corneal lesion, once having reached its maximum, tends to regress somewhat, although the total number of organisms present does not decrease. Following the original inoculation, a relative immunity rapidly develops so that a second inoculation, into the other cornea, no longer produces a lesion and the organisms show no evidence of multiplication. It is believed that this immunity is responsible for the fact that a primary inoculum produces only a limited corneal infection with no systemic spread of the disease (Rees and Robson, 1950; Robson and Sullivan, 1957; Robson, Sullivan and Didcock, 1957).

When a mouse receiving a primary inoculum into the cornea is treated with cortisone from the time of inoculation, the infection no longer remains limited but spreads to involve the whole eye, and also systemically, so that the animal ultimately dies with generalized tuberculosis (Naguib and Robson, 1955). Examination of the cornea of such an animal with the phase contrast microscope shows that the hormone treatment for a time prevents the cellular reaction, so that rapid multiplication and spread of the organisms can occur. When cellular invasion does occur it is no longer effective in dealing with the infection (Robson and Didcock, 1956).

The purpose of the present investigation was to follow quantitatively the multiplication of *M. tuberculosis* inoculated into the cornea of animals treated with cortisone and also to determine the effect of the hormone on the immunity process.

METHODS

Albino mice of the "C" strain were used and were inoculated intracorneally, using the technique of Rees and Robson (1950). The mice were immunized by intracorneal inoculation of *M. tuberculosis, var. bovis* and the challenge infection was given one month later by inoculation into the other cornea, as described by Robson, Sullivan and Didcock (1957).

The immunizing and challenging dose consisted of about 0.01 ml. of a 1/100 dilution of a culture in Dubos medium (approximately 5,000 bacilli). The dilutions were made in normal saline containing 0.05% Tween 80. The animals were divided into four groups:

(1) Animals receiving a primary infection in one cornea.
(2) Animals receiving a primary infection and treated with cortisone.
(3) Animals receiving a reinfection in the second cornea 30 days after the primary infection.
(4) Animals receiving a reinfection and treatment with cortisone.

For convenience, these groups are referred to as "normal," "normal plus cortisone," "immune," and "immune plus cortisone," respectively.

In those groups of animals which received cortisone, treatment of immune animals was started on the day before reinoculation and that of animals receiving only the primary infection on the day before inoculation.

All the animals were given aureomycin (0.5 g./l.) in the drinking water to prevent secondary infection. The adrenal hormone treatment was given both systemically and locally, as cortisone acetate 0.5 mg./day subcutaneously and hydrocortisone eye drops 10 mg./ml. twice daily (Roussel). The animals not receiving cortisone were given the same volumes of suspending fluids by the same routes, namely systemically and locally.

For examination, the animal was killed and the cornea removed and examined by phase contrast microscopy as previously described (Robson and Didcock, 1955). It was then stained as a whole and the number of bacilli present estimated by the method of Robson and Sullivan (1957). This entails comparison of the density of the mass of bacilli in the cornea with a standard scale of densities (the "T" scale), and the results are expressed as "T" units, each of which corresponds approximately to double the number of bacilli of the previous unit.

The area of the cornea over which the bacilli are originally inoculated is approximately 500 μ in diameter and does not change appreciably during the first fortnight of the experiment, though the density of the bacilli over that area increases with their multiplication. The "T" value for a particular cornea can thus be taken as an index of the number of bacilli present, without taking into account the area over which the bacilli are spread. In the animals on cortisone, small groups of bacilli became detached from the main mass and migrated towards the periphery of the cornea, but their number was quite small in comparison with the main mass and thus did not affect the validity of the "T" value as a criterion of the total number of bacilli present in the cornea.

Results

Macroscopically, the corneas of the "normal" animals remained clear until the appearance of a tiny lesion after about 7 days. The lesions gradually increased in size during the following weeks, reaching a maximum at about one month after inoculation. Blebs were only occasionally seen in these eyes.

In the "normal plus cortisone" groups the appearance of lesions was delayed until about the 11th day after inoculation. The formation of blebs also began at this time, and by the 15th day after inoculation more than half of the animals had blebs visible in the cornea. Both the blebs and the surrounding lesions increased in size until the end of the experiment on the 18th day.

In the "immune" group no lesions appeared in the challenged eye throughout the period of observation.

The macroscopic appearance of the "immune plus cortisone" group was almost exactly the same as of the "normal plus cortisone" group, with the development of lesions and blebs, so that the immunity was completely suppressed.

Microscopic examination of the corneas in the four groups showed that in the "normal" groups there was a rapid cell invasion with phagocytosis of the bacilli as previously described (Robson and Didcock, 1955). In the "immune" group there was less invasion of the challenged eye by phagocytes and bacillary multiplication was inhibited (Robson, Sullivan and Didcock, 1957).

The appearances in the "normal plus cortisone" and "immune plus cortisone" groups were exactly the same. There was almost complete inhibition of cell invasion at first and the bacilli multiplied,

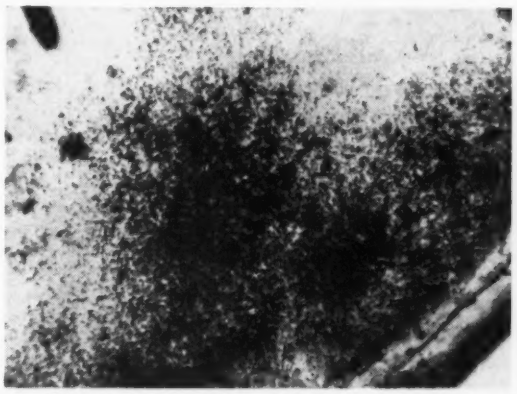

FIG. 1.—Unsectioned mouse cornea 10 days after inoculation with *M. tuberculosis, var. bovis*. Stained with carbol fuchsin and counterstained with methylene blue. Note cell invasion. The bacilli, stained red in the original preparation, were seen dispersed in and among the cells, but are not obvious in this photograph. (×240.)

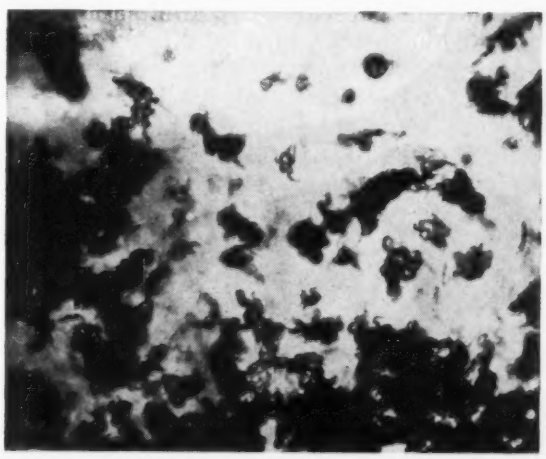

FIG. 2.—Unsectioned mouse cornea 10 days after inoculation with the same inoculum as in Fig. 1. Mouse treated daily with 0.5 mg. of cortisone. Stained with carbol fuchsin and counterstained with methylene blue. Note massive cords of bacilli and complete absence of cell invasion. (×240.)

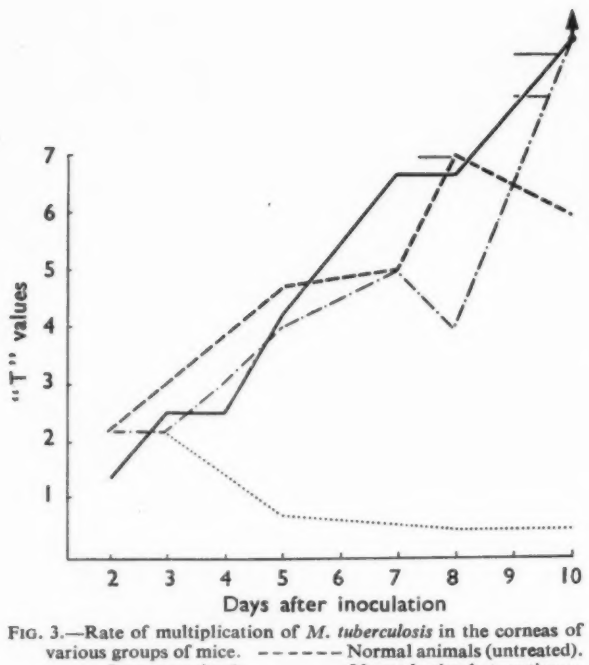

FIG. 3.—Rate of multiplication of *M. tuberculosis* in the corneas of various groups of mice. – – – – – – Normal animals (untreated). . . . . Immune animals. ——— Normal animals + cortisone. –.–.–. Immune animals + cortisone. Arrow indicates a value well above the T7 level to which no accurate figure can be given.

forming cords and palisades. In a few animals, where some leucocytes and macrophages had invaded the cornea, there seemed to be little or no attempt at phagocytosis, so that macrophages not containing any bacilli were often seen close to small groups of organisms. Furthermore, there was no aggregation of corneal corpuscles around the site of the bacilli, as is seen in untreated animals. The appearances of the stained cornea in an untreated animal and in an animal treated with cortisone are shown in Figs. 1 and 2. As previously reported (Robson and Didcock, 1956), cellular invasion of the cornea is not permanently inhibited under the influence of cortisone and this was strikingly seen in the present experiment. On the 15th day after inoculation only a few intracellular bacilli were seen, but 2 days later there was marked cellular invasion, forming an impenetrable core of cells and bacilli.

The increase in the number of bacilli in the corneas of the various groups, as estimated by the "T" scale, is shown in Fig. 3. Animals were killed at various times after inoculation and each value on the graph represents the average of the "T" values of three corneas. No bacillary multiplication was observed in the "immune" group, which is in agreement with previous observations (Robson, Sullivan and Didcock, 1957).

There was rapid multiplication in the other groups, and, during the first 8 days or so after inoculation, the rate of multiplication was the same in all of the three groups. After this period, however, multiplication in the "normal" group slowed down, whereas in the cortisone-treated animals the multiplication continued unimpeded until the end of the experiment, by which time the cornea was packed with massive palisades of bacilli.

Systemic Disease.—On the 18th day after inoculation, all the remaining animals were killed and post-mortems carried out to search for evidence of systemic disease. The animals were examined macroscopically and smears of the liver and lung were stained with Ziehl-Neelsen stain. No sections were cut except in one case of a lung nodule which proved to be a typical tuberculous focus. In the "normal" and "normal plus cortisone" groups no evidence of systemic disease was found and no acid-fast bacilli were found.

In one animal in the "immune" group acid-fast organisms were found in a hard yellow nodule attached to the under-surface of the liver.

In the "immune plus cortisone" group 7 of 8 animals had macroscopic evidence of infection, and in 5 of these acid-fast organisms were found in smears of the lungs.

Effect of Cortisone on an Established Lesion.—The right (immunizing) eyes of the two immune groups were removed and examined when the experiment was terminated, namely, 7 weeks after infection (18 days after reinfection of the second eye, and after 19 days of cortisone treatment). In corneas from 5 "immune" animals there were large masses of bacilli corresponding to the large macroscopic lesions. Nearly all the bacilli were intracellular and there was little evidence of spread into the sclera. No cord formation was seen in any of these animals. In all 8 animals of the "immune plus cortisone" groups examined, extracellular bacilli were seen, and clear evidence of cord formation and spread to the sclera was seen in 5 of these animals.

Thus the administration of cortisone to animals with established lesions caused a reactivation of the infection with free extracellular multiplication of the bacilli and spread of the infection.

DISCUSSION

These experiments show that treatment with cortisone does not affect the initial rate of multiplication of tubercle bacilli in the cornea of mice. For the first 7 days after inoculation the number of organisms increased at the same rate in the control and cortisone-treated animals. Since in the control

mice the bacilli rapidly became intracellular, whereas in the treated animals they remained extracellular, multiplying in cords, throughout this period, it can be concluded that, under the conditions of these experiments, they multiply at the same rate inside leucocytes as in an extracellular environment. These results are also in agreement with the finding that cortisone does not enhance the multiplication of *M. tuberculosis in vitro* (Lurie, Zappasodi, Dannenberg, and Cardona-Lynch, 1953).

After the 7th day the bacilli in the control corneas show little further multiplication, presumably because of the development of immunity. It has been shown that, if at this stage the second cornea of such an animal is challenged, little or no bacillary multiplication occurs (Robson, Sullivan and Didcock, 1957). In the cortisone-treated animals, on the other hand, multiplication continues, leading to the formation of very thick cords of bacilli with destruction of the eye and ultimate systemic spread and death, as shown by Naguib and Robson (1955). This shows that massive cord formation can occur not only *in vitro* but also in living tissues. It seems likely that these changes occur because the immunity, which checks the further multiplication in the control animals, is not effective in the presence of cortisone. There are two possible explanations for this: (1) The treatment with cortisone has prevented the development of immunity. There is indeed some evidence that cortisone inhibits the formation of antibodies during active immunization (Fischel, 1953), though the effect is by no means a complete one (Germuth, 1956). In any case, the question as to whether antibodies play any part in immunity to tuberculosis is by no means settled. (2) Immunity has developed but is unable to exert its action. If immunity in tuberculosis is dependent on the action of cells on the bacilli, as has been suggested, then the inhibition of cellular invasion which is observed would explain the failure of the immune process to check the bacillary multiplication.

Furthermore, it has been observed that there is a positive correlation between cord formation *in vitro* and virulence (Middlebrook, Dubos and Pierce, 1947). It may well be that the massive cord formation seen in the corneas of cortisone-treated animals allows the development of an increased virulence of the bacilli, and this may also be responsible, to some extent, for the eventual spread and fatal outcome of the disease.

Solotorovsky, Gregory and Stoerk (1951) have shown that cortisone overcomes the beneficial effects of vaccination with heat-killed tubercle bacilli in mice inoculated intravenously with a highly virulent strain of *M. tuberculosis*. These results are in agreement with the previous findings of Hart and Rees (1950), who found that cortisone exacerbated a chronic tuberculous infection in mice, presumably overcoming the immunity present in these mice. In the present experiments, an immunity sufficient to inhibit completely the increase in the number of organisms in the challenged cornea was overcome so that the organisms multiplied at the same rate as in an eye not protected by previous immunization. Since cellular invasion was almost completely inhibited in the cortisone-treated animals, these results could be explained on the basis of a cellular mechanism of immunity in tuberculosis. It must, however, be remembered that cortisone not only inhibits the production of antibodies during active immunization, but also after immunization is well established (Fischel, 1953), so that these results do not exclude the possibility that the effect of cortisone involves some interference with antibodies.

The effect of cortisone on the immunizing infection in the first cornea also requires consideration. In a number of these animals there was clear extracellular bacillary multiplication, with cord formation and spread of bacilli to the sclera, and the development of systemic disease. This is not seen in corresponding animals not treated with cortisone, in which no evidence of bacillary multiplication is observed. This difference may be dependent on the comparatively short life of macrophages. When such cells break down in untreated animals, the bacilli released are taken up by new macrophages and, in the presence of immunity, no further bacterial multiplication occurs. In the cortisone-treated animals, new macrophages are not available to take up the released bacilli, which multiply, invade the sclera and spread systemically. This seems to be the most reasonable explanation for the phenomena observed.

We are grateful to the Medical Research Council for a grant for expenses (to J.M.R.); to Guy's Hospital Endowment Fund for a personal grant (to F.M.S.) and for technical assistance; to Mr. K. A. H. Didcock, who collaborated in the preliminary investigation of this problem; and to Dr. J. J. Segall, of Roussel Laboratories, for a supply of hydrocortisone drops.

References

Fischel, E. E. (1953). In *The Effect of ACTH and Cortisone upon Infection and Resistance* (G. Shwartzman, ed.), p. 56. Columbia University Press.

Germuth, F. G. (1956). *Pharmacol. Rev.*, **8**, 1.

Hart, P. D'Arcy, and Rees, R. J. W. (1950). *Lancet*, **2**, 391.

Lurie, M. B., Zappasodi, P., Dannenberg, A. M. Jr. and Cardona-Lynch, E. (1953). In *The Effect of ACTH and Cortisone upon Infection and Resistance* (G. Shwartzman, ed.), p. 84. Columbia Universtiy Press.

Middlebrook, G., Dubos, R. J., and Pierce. C. (1947). *J. exp. Med.*, **86**, 175.

Naguib, M., and Robson, J. M. (1955). *Brit. J. Pharmacol.*, **10**, 387.

Rees, R. J. W., and Robson, J. M. (1950). Ibid., **5**, 77.

Robson, J. M., and Didcock, K. A. H. (1955). *Brit. J. exp. Path.*, **36**, 560.

—— —— (1956). *Amer. Rev. Tuberc.*, **74**, 1.

—— and Sullivan, F. M. (1957). Ibid., in the press.

—— —— and Didcock, K. A. (1957). *Brit. J. exp. Path.*, in the press.

Solotorovsky, M., Gregory, F. J., and Stoerk, H. C. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 286.

Brit. J. Pharmacol. (1957), **12**, 245.

THE RELATION BETWEEN SEDATION AND BODY TEMPERATURE IN THE MOUSE

BY

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Chlorpromazine and reserpine reduce locomotor activity and prolong pentobarbitone hypnosis in mice. Both these effects are shown to be proportional to the fall in body temperature produced by these drugs. Other agents are shown to reduce body temperature and potentiate pentobarbitone. At ambient temperatures of 32° C. neither chlorpromazine nor reserpine is hypothermic or sedative. It is concluded that sedative effects in the mouse at ordinary room temperatures are related to the hypothermic properties of these drugs. At 36° C., while reserpine fails to potentiate pentobarbitone, chlorpromazine still does so.

Sedation may be recognized as a condition of reduced activity which can be observed following the administration of hypnotics, such as the barbiturates, in sub-hypnotic doses. Chlorpromazine and reserpine are characterized by their ability to produce sedation, whilst being devoid of hypnotic properties. Both drugs potentiate the hypnotic effect of barbiturates in the mouse (Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet, 1953; Kopera and Armitage, 1954; Cronheim and Toekes, 1954) and this property has been regarded as an expression of sedative action.

Lowered body temperature has been shown to prolong pentobarbitone anaesthesia (Fuhrman, 1947), and since chlorpromazine and reserpine cause hypothermia (Courvoisier *et al.*, 1953; Kopera and Armitage, 1954; Plummer, Earl, Schneider, Trapold, and Barrett, 1954) it seemed possible that these two properties were related. This possibility was supported by the evidence that various agents which lower body temperature, such as histamine, antihistamines, and pethidine (Packman, Rossi, and Harrisson, 1953; Dutta, 1948) also potentiate barbiturate anaesthesia (Winter, 1948; Ambrus, Ambrus, Leonard, Moser, and Harrisson, 1952; Kopera and Armitage, 1954).

In this paper we have investigated the relationship between body temperature and sedation, as measured by reduction in activity and potentiation of pentobarbitone hypnosis by sedative agents, in the mouse.

METHODS

Rectal temperatures were measured using a probe consisting of a type F thermistor mounted in a Perspex holder and registering directly on a microammeter in a circuit similar to that described by Grieve (1951). The probe was inserted 2 cm. into the rectum. Groups of 5 or 10 mice were used and the mean temperature recorded. At body temperatures of about 38° C., this method gave readings with a standard deviation ±0.6° C., increasing with lower temperatures, reaching ±1° C. at 35° C. and ±2° C. at 29° C.

Activity was measured by interruption of a beam of light, by a modification of the method of Dews (1953). Mice were placed singly in a Perspex counting chamber 13 cm. square for a period of 15 min., the scores being registered automatically. Rectal temperature readings were taken immediately before and after activity measurement and the mean recorded.

Sleeping time was taken as the period between injection of a mouse with pentobarbitone and the return of its ability to right itself when disturbed.

The room temperature was maintained at 22° C.; experiments at higher temperatures were performed in an incubator. Male albino mice of 16 to 24 g. weight were used. The mice were not starved before use; food and water were withheld during experiments lasting less than 6 hr. Pentobarbitone sodium was injected intravenously and all other drugs intraperitoneally, in volumes of 0.2 ml./20 g. All drugs were dissolved in saline with the exception of reserpine, which was dissolved in 2N acetic acid, neutralized as far as possible and diluted with water.

RESULTS

The Effect of Chlorpromazine and Reserpine on Body Temperature.—Chlorpromazine and reserpine caused a fall in rectal temperature of mice, which reached minimum levels 1½ to 3 hr. after injection of chlorpromazine (2 to 5 mg./kg.) (Fig. 1, B) and 4 to 8 hr. after reserpine (0.5 to 5 mg./kg.) (Fig. 2, B). Fig. 3 shows the minimum mean rectal temperature reached by groups of mice

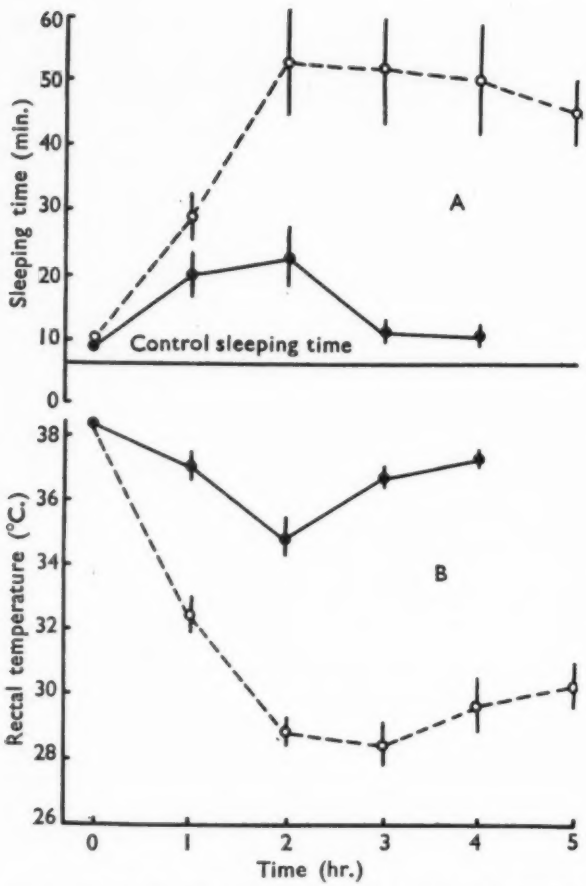

FIG. 1.—A. Effect of chlorpromazine 3 and 5 mg./kg. i.p., injected at 0, on the mean sleeping time of groups of 10 mice treated after various intervals with 25 mg./kg. pentobarbitone i.v. The sleeping time produced by the dose of pentobarbitone in normal mice is indicated. B. Mean rectal temperatures of these groups of mice taken immediately before injection of pentobarbitone. In both graphs, ●——●=3 and ○- - -○=5 mg./kg. chlorpromazine. The vertical lines represent standard errors of mean values. Room temperature, 22° C.

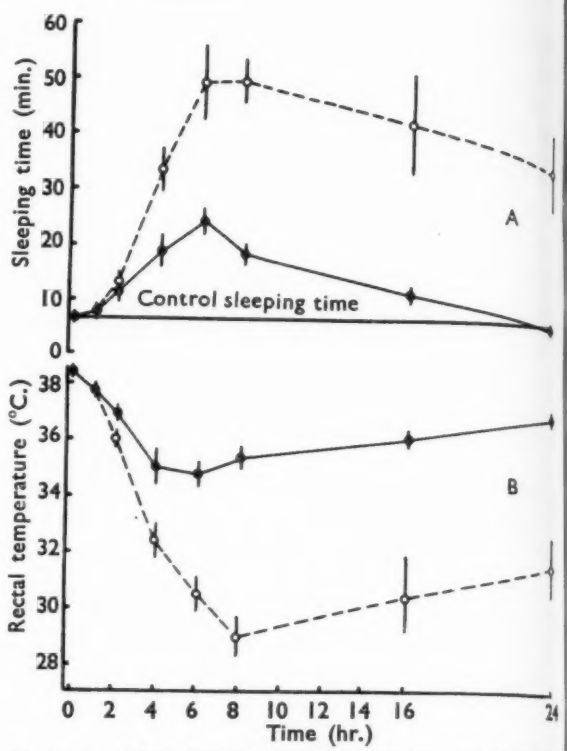

FIG. 2.—A. Effect of reserpine 1.0 and 1.5 mg./kg. i.p., injected at 0, on the mean sleeping time of groups of 10 mice treated after various intervals with 25 mg./kg. pentobarbitone i.v. The sleeping time produced by the dose of pentobarbitone in normal mice is indicated. B. Mean rectal temperatures of these groups of mice taken immediately before injection of pentobarbitone. In both graphs, ●——●=1.0 and ○- - -○=1.5 mg./kg. reserpine. The vertical lines represent standard errors of mean values. Room temperature, 22° C.

treated with chlorpromazine or reserpine, plotted against the logarithm of the dose. Temperatures are expressed as differences from controls.

There was a clear regression of temperature fall upon dose with each drug; no fall was produced by doses of less than 1.5 mg./kg. chlorpromazine or 0.3 mg./kg. reserpine.

Activity and Body Temperature.—Locomotor activity was measured in mice after injection of 0.25 to 2 mg./kg. chlorpromazine or reserpine. Measurements were made 30 min. to 2 hr. after chlorpromazine and 1 to 4 hr. after reserpine. The lowest doses were included to determine whether these drugs could reduce activity without lowering body temperature.

Activity counts varied from 10 to 160/15 min., with mean rectal temperatures of 35.0 to

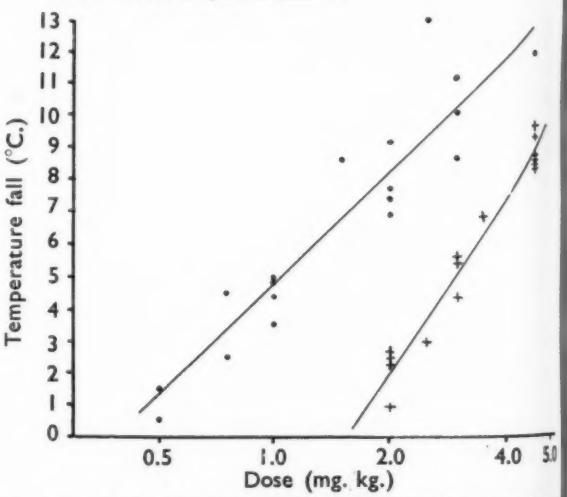

FIG. 3.—The effect of chlorpromazine and reserpine, given intraperitoneally, upon the rectal temperature of mice. Each value for temperature fall is the lowest recorded for a group of 5 mice after injection of the drug, and is expressed as the difference from the mean value for an untreated group. ●=Reserpine; b=12.0±1.9 (P=0.95); +=Chlorpromazine; b=17.8±2.1 (P=0.95).

39.0° C. A fall in rectal temperature to 34° C. was sufficient to abolish locomotor activity in the mouse. The temperature values were plotted against log. activity counts (Fig. 4) and correlation was apparent for each drug, as follows:

Chlorpromazine .. r=0.85 (73 readings)
Reserpine .. .. r=0.81 (56 readings)

Correlation was highly significant ($P<0.001$) in both cases. The regression lines did not differ significantly from each other in slope or position ($P>0.3$).

Untreated mice had rectal temperatures of 37.25 to 39.0° C. and activities of 50 to 170 counts per 15 min. There was little or no correlation (r=0.03), but it may be seen that the area enclosing the standard deviations of temperature and activity about the means for untreated mice was distributed about the upper ends of the regression lines for those treated with the drugs.

Potentiation of Pentobarbitone and Body Temperature.—The prolongation produced by 2 to 5 mg./kg. chlorpromazine and 0.5 to 3 mg./kg. reserpine upon the duration of pentobarbitone hypnosis increased with the interval between injection of the potentiating drug and the barbiturate (Figs. 1, A and 2, A), reaching a maximum 2 to 3 hr. after chlorpromazine and 6 to 8 hr. after reserpine.

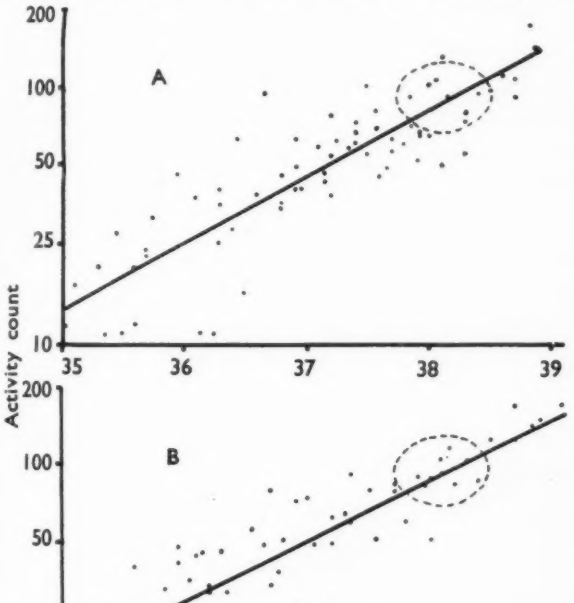

FIG. 4.—Rectal temperatures and activity counts for mice treated with (0.25 to 2 mg./kg.): A, chlorpromazine, and B, reserpine. Slope A=0.25±0.04 (P=0.95), 73 values; B=0.22±0.044 (P=0.95), 56 values. The area within the ellipse represents the standard deviations about the means of temperature and activity in 34 untreated mice. Room temperature, 22° C.

The time course for the hypothermia produced by each of these two drugs was followed, and Figs. 1, B and 2, B show that the curves for change in body temperature reflect the corresponding curves for barbiturate potentiation, minimum temperatures coinciding approximately with maximum prolongation of sleeping time.

A relation was clearly apparent between corresponding values for the two effects, and this was further examined, by using values for single mice. These were injected with 1.0 to 5.0 mg./kg. chlorpromazine or 0.5 to 3 mg./kg. reserpine, and, after the appropriate interval for maximum effect on body temperature, the rectal temperature was measured and 25 mg./kg. pentobarbitone injected. The resulting sleeping time for each mouse was plotted against its rectal temperature (Fig. 5). Significant correlation between temperature and subsequent sleeping time was found for both drugs (chlorpromazine −0.74, reserpine −0.9; $P<$ 0.001). The two regression lines could not be shown to differ significantly in slope or position, and both passed through values for temperature and sleeping time in mice treated only with pentobarbitone.

Among other substances found to lower rectal temperature and prolong pentobarbitone hypnosis were 5-hydroxytryptamine, for which potentiation of barbiturate has already been reported (Shore, Silver, and Brodie, 1955), adenosinetriphosphate, which is known to lower body temperature (Green and Stoner, 1950), and promethazine, for which both properties have been described (Courvoisier *et al.*, 1953; Packman *et al.*, 1953; Kopera and Armitage, 1954). Doses of these agents causing a similar decrease in temperature (Fig. 5) were found to prolong the sleeping time to the same extent (Table I). Intraperitoneal injections of saline, used in control experiments, were without effect on body temperature or pentobarbitone sleeping time.

Influence of External Temperature.—It has been shown that chlorpromazine in the rat (Maier, Forster, Schaff, and Kayser, 1955) and reserpine in the rabbit (Plummer *et al.*, 1954) do not reduce body temperature when the ambient temperature is about 31° C. (thermal neutrality), and this has been taken as evidence that these drugs lower body temperature by interfering with temperature regu-

FIG. 5.—Rectal temperatures and sleeping times for mice treated with: A, 1.0 to 5.0 mg./kg. chlorpromazine, and B, 0.5 to 3 mg./kg. reserpine, followed by 25 mg./kg. pentobarbitone given 1 to 3 hr. after chlorpromazine and 4 to 6 hr. after reserpine. The temperature of each mouse was taken immediately before injecting the pentobarbitone. Room temperature, 22° C. Slope A= -5.32 ± 1.4 ($P=0.95$), 54 values; B= -4.91 ± 0.63 ($P=0.95$), 60 values. The area within the ellipse represents the standard deviations about the means of temperature and sleeping time in 20 mice treated only with 25 mg./kg. pentobarbitone. The sleeping times shown on the left-hand ordinate are produced in untreated mice by the doses of pentobarbitone shown on the right-hand ordinate.

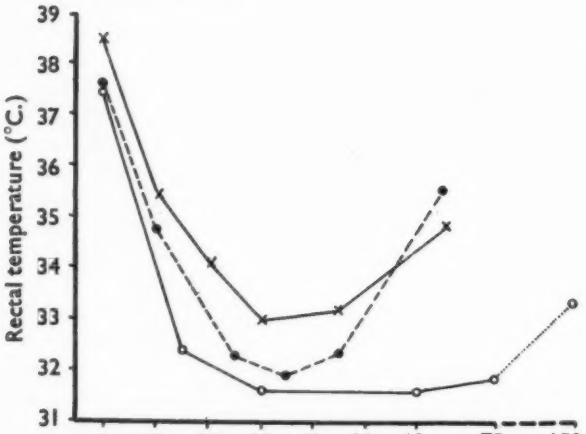

FIG. 6.—The effect of various agents on the mean rectal temperature of groups of mice. ×——×=5-hydroxytryptamine creatinine sulphate, 100 mg./kg. ○——○=promethazine hydrochloride, 30 mg./kg. ●- - -●=adenosinetriphosphate, 200 mg./kg. Room temperature, 22° C.

lation. We have made similar observations in the mouse and have found that chlorpromazine and reserpine also produce no apparent sedation under these conditions. Both, however, prolong pentobarbitone anaesthesia at 32° C. (Table II). It was further found that pentobarbitone lowered body temperature at 32° C.; this effect was also prolonged by chlorpromazine and reserpine. At 36° C. the body temperature cannot fall and reserpine did not prolong pentobarbitone hypnosis at this temperature; chlorpromazine, however, exerted considerable potentiation (Table II).

TABLE I

THE EFFECT OF 5-HYDROXYTRYPTAMINE CREATININE SULPHATE, PROMETHAZINE AND ADENOSINETRIPHOSPHATE UPON THE SLEEPING TIME DUE TO 35 MG./KG. PENTOBARBITONE

Room temp. 22° C.

Pentobarbitone was injected 5 min. after the 5-hydroxytryptamine or adenosinetriphosphate and 45 min. after the promethazine. These intervals were chosen by reference to the period required for maximum effect of these drugs upon body temperature (Fig. 5).

	Sleeping Time (min.)±S.E.	
	Treated	Controls
5-Hydroxytryptamine, 100 mg./kg.	37·3±3·03	11·5±1·1
Promethazine, 30 mg./kg. ..	36·6±3·25	11·8±1·5
Adenosinetriphosphate, 200 mg./kg.	50·7±6·0	14·2±2·3

DISCUSSION

The results of activity measurement suggest a relationship between sedation and body temperature, since reduction in activity by both chlorpromazine and reserpine is proportional to the fall

TABLE II

THE DURATION OF PENTOBARBITONE HYPNOSIS IN MICE TREATED WITH RESERPINE OR CHLORPROMAZINE AT VARIOUS TEMPERATURES

Numerals in parenthesis denote the number of mice used. The sleeping times with chlorpromazine 3 mg./kg. all show significant potentiation ($P<0.001$). For reserpine 1 mg./kg., at 22° C. and 32° C., $P<0.001$, and at 36° C., $P\sim0.15$.

Room Temp. (°C.)	Pentobarbitone (mg./kg.) i.v.	Sleeping Time (min.)±S.E.		
		Control	2 Hr. after 1 mg./kg. Reserpine i.p.	1 Hr. after 3 mg./kg. Chlorpromazine i.p.
22	25	7·9±0·9 (15)		24·0±2·4 (10)
22	35	11·8±1·5 (10)	26·3±2·9 (10)	
32	40	17·6±1·8 (10)	34·3±3·5 (10)	41·2±1·7 (10)
36	50	21·0±2·4 (10)	25·5±1·5 (10)	69·3±4·6 (10)

in rectal temperature which occurs. Moreover, the relationship appears to be the same for both these drugs. Neither chlorpromazine nor reserpine reduced activity significantly below the range found in untreated mice unless temperature was also reduced. It is therefore possible that these sedatives reduce activity in the mouse by virtue of their ability to lower body temperature.

This possibility is supported by the absence of apparent sedation in mice treated with chlorpromazine or reserpine at an ambient temperature of 32° C., at which these drugs caused no fall in body temperature.

A relationship has also been shown between the fall in temperature produced by the hypothermic drugs and their ability to prolong pentobarbitone hypnosis at room temperature. The correlation found between rectal temperature and sleeping time, and the similarity in this relationship for chlorpromazine and reserpine, suggests that their potentiation of hypnosis at normal environmental temperatures may be due to the fall in body temperature. In support of this, no evidence of potentiation could be found with doses of chlorpromazine or reserpine which failed to lower body temperature. Furthermore, a similar time course has been shown for both effects, minimum body temperature and maximum potentiation of pentobarbitone occurring together.

The variation in duration of barbiturate hypnosis with room temperature, reported by Raventós (1938) and others, may be explained by its dependence upon body temperature. Fuhrman (1947) related this to reduction by lower temperature of the activity of the enzyme responsible for detoxication, by showing that barbitone, which is largely excreted unchanged, was equally effective at different body temperatures. The dependence of detoxication rate upon temperature is also adequate to explain the prolongation of barbiturate hypnosis exerted by hypothermic agents at room temperature. Reference to Fig. 5 shows that a fall in body temperature of 10° C. increased sleeping time from 7 to 60 min. In terms of effective dose of pentobarbitone, taken from the dose-response curve, this represents the conversion of 25 mg./kg. to the equivalent of 65 mg./kg., that is, an increase by about 2.5-fold. This value is of the order which might be expected for the Q_{10} of a chemical reaction such as that responsible for the detoxication of pentobarbitone (Brodie, Burns, Mark, Lief, Bernstein, and Papper, 1953).

It may be suggested, therefore, that reserpine and chlorpromazine produce, by lowering body temperature, a reduction in the rate of detoxication. They would, on this supposition, have this mode of action in common with the potentiating agent β-diethylaminoethyl diphenylpropyl acetate (SKF 525A) (Cook, Toner, and Fellows, 1954), which, however, has been shown to inhibit the detoxicating enzyme system directly (Axelrod, Reichenthal, and Brodie, 1954) and shows no sedative action.

The results at 32° C. do not appear to bear out the relation between hypothermia and barbiturate potentiation suggested by the results at 22° C., for neither reserpine nor chlorpromazine are hypothermic at the higher temperature, whereas both prolonged pentobarbitone hypnosis. However, reserpine failed to potentiate the barbiturate at 36° C., at which no fall of body temperature can occur. In addition, it was found that pentobarbitone itself reduced the body temperature of mice at 32° C., and this effect was prolonged by reserpine. It may be concluded, therefore, that potentiation of barbiturate hypnosis by reserpine does not occur unless the body temperature falls.

This is certainly not true of chlorpromazine, which potentiated pentobarbitone hypnosis at both 32° C. and 36° C. At these temperatures, the action of this drug cannot be related to a hypothermic effect and represents an alternative means of potentiation which does not, however, appear to contribute to the potentiating action of chlorpromazine at lower room temperatures.

REFERENCES

Ambrus, J. L., Ambrus, C. M., Leonard, C. A., Moser, C. E., and Harrisson, J. W. E. (1952). *J. Amer. pharm. Ass.*, **41**, 606.

Axelrod, J., Reichenthal, J., and Brodie, B. B. (1954). *J. Pharmacol.*, **112**, 49.

Brodie, B. B., Burns, J. J., Mark, L. C., Lief, P. A., Bernstein, E., and Papper, E. M. (1953). Ibid., **109**, 26.

Cook, L., Toner, J. J., and Fellows, E. J. (1954). Ibid., **111**, 131.

Courvoisier, S., Fournel, J., Ducrot, R., Kolsky, M., and Koetschet, P. (1953). *Arch. int. Pharmacodyn.*, **92**, 305.

Cronheim, G., and Toekes, I. M. (1954). *Fed. Proc.*, **13**, 345.

Dews, P. B. (1953). *Brit. J. Pharmacol.*, **8**, 46.

Dutta, N. K. (1948). Ibid., **3**, 246.

Fuhrman, F. A. (1947). *Science*, **105**, 387.

Green, H. N., and Stoner, H. B. (1950). *Biological Actions of the Adenine Nucleotides.* London: H. K. Lewis.

Grieve, J. (1951). *Lancet*, **2**, 431.

Kopera, J., and Armitage, A. K. (1954). *Brit. J. Pharmacol.*, **9**, 392.

Maier, A., Forster, E., Schaff, G., and Kayser, Ch. (1955). *C.R. Soc. Biol., Paris*, **149**, 568.

Packman, E. W., Rossi, G. V., and Harrisson, J. W. E. (1953). *J. Pharm., Lond.*, **5**, 301.

Plummer, A. J., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W. (1954). *Ann. N.Y. Acad. Sci.*, **59**, 8.

Raventós, J. (1938). *J. Pharmacol.*, **64**, 355.

Shore, P. A., Silver, S. L., and Brodie, B. B. (1955). *Experientia*, **11**, 272.

Winter, C. A. (1948). *J. Pharmacol.*, **94**, 7.

Brit. J. Pharmacol. (1957), **12**, 251.

PROLONGATION OF CHLORAL HYDRATE SLEEPING TIME BY 5-HYDROXYTRYPTAMINE AND BY CERTAIN OTHER DRUGS

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Various drugs have been tested for a capacity to prolong the hypnotic effect of chloral hydrate in mice. Amongst the compounds which, when injected subcutaneously in substantial amount shortly before the chloral hydrate (250 mg./kg. intraperitoneally), increased sleeping time significantly were adrenaline, noradrenaline, phenylephrine, methoxamine, 5-hydroxytryptamine (serotonin), histamine, ergotamine, yohimbine, and atropine. The ability of these drugs to prolong chloral hydrate sleeping time could not be related to any common circulatory property, but most of the active drugs are known to lower body temperature under comparable conditions. It was found that mice which have been pre-treated with 5-hydroxytryptamine or adrenaline suffer a much greater fall of body temperature when chloral hydrate is given subsequently than do mice which have been given chloral hydrate alone. It is suggested that some, at least, of the drugs which prolong the effects of hypnotics do so by virtue of a hypothermic action.

Mice which have received 5-hydroxytryptamine (5-HT) sleep longer than normal animals when given a fixed dose of a barbiturate (Shore, Silver, and Brodie, 1955; Fornaroli and Koller, 1955; Fastier, 1956). While some substances can prolong the effects of barbiturates by interfering with their enzymatic inactivation, the sleep-prolonging action of 5-HT seemed unlikely to be due to an action of this type because it can extend to the hypnotic action of chloral hydrate which is not metabolized in the same way as barbiturates. Experiments bearing on the mechanism of action have been discussed by Shore *et al.* (1955), Salmoiraghi, Sollero, and Page (1956), Fastier (1956), and Richards and Taylor (1956).

As the hypnotic effect of chloral hydrate in mice can be prolonged, not only by 5-HT and such nearly related compounds as tryptamine and bufotenine, but also by such agents as adrenaline and histamine, Fastier (1956) suggested that the sleep-prolonging effect of 5-HT might be due to a relatively non-specific vascular action. Like Werle and Lentzen (1938), Fastier (1956) was impressed by the fact that many of the drugs which have been shown to prolong the effects of hypnotics (under some conditions at least) are vaso-active drugs.

This paper reports the results of some experiments seeking to establish a connexion between effects on the circulatory system and hypnosis-prolonging activity. It seemed possible that some compounds might prolong effects of certain hypnotics by vascular mechanisms affecting, for example, the absorption of the drug from the site of injection, its penetration of the "blood-brain barrier," or its breakdown or excretion. Since Fuhrman (1946) has demonstrated that the lowering of body temperature by 10° C. can increase pentobarbitone sleeping time more than three-fold, experiments were also carried out to see whether pre-treatment with 5-HT or adrenaline could result in a much greater fall of body temperature than usual when a hypnotic was given.

METHODS

The dose of chloral hydrate usually employed (250 mg./kg.) was that which put mice to sleep for about 30 min. Chloral hydrate (1 g.) was dissolved in half-isotonic saline (40 ml.), whether used for intraperitoneal or intravenous injection. Other drugs were generally given subcutaneously, to prevent interference with the absorption of chloral hydrate. After the mice had fallen asleep, they were placed a short distance apart from one another, so that a waking mouse would disturb its neighbours as little as possible. The waking time was taken as that at which a mouse righted itself and began to move away from its sleeping position. The transition from sleeping to waking appeared to be very quick. Mice which

TABLE I

EFFECT OF ADRENALINE AND RELATED DRUGS, AND OF 5-HT, ON THE DURATION OF HYPNOSIS PRODUCED IN MICE BY THE INTRAPERITONEAL INJECTION OF CHLORAL HYDRATE (250 MG./KG.)

Each compound was injected either subcutaneously (s.c.) or intraperitoneally (i.p.) 10 min. before the chloral hydrate. The mice in each control group were given an injection of 0.9% NaCl by the same route 10 min. before the chloral hydrate. The sleeping times given are the means for groups of 10 mice. The numerals in brackets after each mean sleeping time give the standard deviation. The *t* test shows a significant ($P<0.01$) increase for the results marked + and a significant ($P<0.01$) decrease for the results marked −. Some earlier results of Fastier (1956) are included for comparison, these experiments being indicated by an asterisk.

Pre-treatment			Sleeping Time (min.)		
Drug	Dose in mg./kg.	Route	Treated Group	Control Group	
Adrenaline* ..	5	i.p.	98 (39)	35 (17)	+
,, ..	5	s.c.	70 (58)	20 (10)	+
,, ..	3	i.p.	65 (29)	24 (6·9)	+
,, ..	3	s.c.	39 (12)	31 (15)	
Noradrenaline ..	5	,,	73 (33)	27 (19)	+
,, ..	3	,,	110 (59)	23 (8·3)	+
,, ..	2	,,	55 (21)	38 (19)	
Phenylephrine ..	50	,,	69 (32)	34 (6·9)	+
,, ..	20	,,	53 (17)	45 (11)	
Methoxamine ..	20	,,	64 (4·8)	21 (1·6)	+
,, ..	20	,,	56 (14)	18 (5·0)	+
Naphthazoline ..	20	,,	75 (27)	24 (8·3)	+
Isoprenaline ..	10	,,	77 (46)	28 (15)	+
,, ..	5	,,	50 (27)	34 (20)	
,, ..	5	,,	41 (6·6)	55 (6·8)	
Amphetamine ..	20	,,	0	29 (4·1)	−
Methylamphetamine	20	,,	0	31 (3·2)	−
5-HT*	20	i.p.	86 (36)	20 (6·9)	+
,, ...	20	s.c.	70 (18)	28 (11)	+
Tryptamine* ..	20	i.p.	37 (9·3)	22 (7·2)	+
Bufotenine* ..	20	,,	81 (31)	22 (8·4)	+

had awakened were removed from the boxes of sleeping mice to avoid their disturbing those left.

The effect of 5-HT on the mydriatic action of hexamethonium in the mouse was studied by measuring pupil size directly by means of a low-power microscope with a scalar eyepiece as described by Blackman, Fastier, Patel, and Wong (1956).

Rectal temperature in mice was measured by inserting a glue-covered constantan-copper thermocouple into the rectum to a fixed distance. Mice were cooled by placing them inside a large refrigerator for a fixed period.

RESULTS

Effects of Various Drugs on Duration of Sleep.—A number of sympathomimetic amines were tested for any obvious relationship between vasoconstrictor activity and a capacity to prolong the hypnotic effect of chloral hydrate in mice (Table I). The response to noradrenaline was found to resemble that to adrenaline. The chloral hydrate sleeping time was also substantially increased by phenylephrine (Neo-Synephrine), methoxamine (Vasoxine), and naphthazoline (Privine), sympathomimetic amines in which the vasoconstrictor and similar "excitatory" actions of adrenaline are most prominently displayed. Isoprenaline, in which the vasodilator and similar inhibitory properties of adrenaline predominate, did not affect chloral hydrate sleeping time significantly when given in the same dose as adrenaline, but it prolonged the chloral hydrate sleeping time considerably when given in the near-lethal dose of 10 mg./kg. Sympathomimetic amines like amphetamine and methylamphetamine (Methedrine), with effects predominantly on the central nervous system, antagonized the hypnotic action of chloral hydrate.

When a wider range of compounds was examined, however, no correlation between vasoconstrictor activity and sleep-prolonging activity was found (Table II). The chloral hydrate sleeping time was increased by certain drugs whose circulatory effects are quite different from those of adrenaline, such as tolazoline (Priscol), yohimbine, and physostigmine. Tolazoline and yohimbine have anti-adrenaline actions, and physostigmine is an anticholinesterase. Atropine also was found to produce a significant increase in the chloral hydrate sleeping time, while methacholine produced a significant shortening. Posterior pituitary extract (Pitibulin) did not affect sleeping time in the doses tried (0.5, 1.0 unit/kg.).

TABLE II

EFFECT OF VARIOUS DRUGS ON CHLORAL HYDRATE SLEEPING TIME IN MICE

Experiments were performed as indicated in Table I. The chloral hydrate was given intraperitoneally, all other drugs subcutaneously and in approximately isotonic solution. "S-methyl" and "S-decyl" refer to the corresponding *iso*thiouronium salts. The asterisk indicates previous results obtained by Fastier (1956). See Table I for explanation of + and − signs.

Pre-treatment		Sleeping Time (min.)		
Drug	Dose in mg./kg.	Treated Group	Control Group	
2-Aminoheptane ..	20	30 (13)	30 (19)	
S-Methyl	50	36 (7·8)	22 (9·1)	+
,, ...	10	31 (5·3)	34 (2·9)	
S-*n*-Decyl	20	50 (4·4)	44 (5·8)	
Mescaline	10	17 (4·0)	16 (3·4)	
Ergotamine	10	25 (2·7)	18 (2·1)	+
Yohimbine	5	61 (7·7)	31 (2·1)	+
Tolazoline	20	46 (17)	18 (5·0)	
,, ...	10	46 (4·5)	20 (2·8)	+
Diphenhydramine ..	10	40 (6·2)	49 (6·2)	
Physostigmine ..	0·25	59 (4·8)	30 (5·0)	+
Methacholine ..	0·5	25 (4·0)	41 (3·9)	−
Atropine	20	55 (20)	41 (29)	
,, ...	10	20 (2·2)	14 (1·3)	+
Pituitrin	(0·5 units)	13 (9·5)	14 (6·0)	
,, ...	(1 unit)	34 (7·6)	27 (10)	
Histamine*	10	51 (3·9)	26 (4·2)	+
Cortisone	20	22 (2·4)	21 (2·6)	
Deoxycortone acetate	20	16 (1·9)	21 (2·7)	
Pacatal	20	46 (8·1)	30 (4·6)	
,, ...	20	27 (4·9)	20 (6·4)	
Sodium nitrite ..	40	33 (2·8)	25 (4·0)	
,, ,, ..	20	19 (2·3)	27 (4·6)	
Distilled water ..	100	34 (7·3)	31 (3·1)	

Influence of Certain Circulatory Changes.—A drug like 5-HT might influence the absorption of a drug given intraperitoneally a few minutes later by causing strong vasoconstriction in the splanchnic region. To test this idea, experiments were performed in which the hypnotic was given intravenously. As 5-HT, methoxamine and tolazoline all caused about the same increase in sleeping time whether chloral hydrate was given intravenously or intraperitoneally (Table III), the prolongation of sleeping time by these drugs cannot be attributed to delayed absorption of the chloral hydrate.

TABLE III

EFFECT OF PREMEDICATION WITH 5-HT, METHOXAMINE, OR TOLAZOLINE ON THE DURATION OF SLEEP PRODUCED BY CHLORAL HYDRATE IN MICE

Chloral hydrate was given either intraperitoneally (i.p.) or intravenously (i.v.). The other drugs were given subcutaneously in a dose of 20 mg./kg. The numbers of mice in the treated and control groups respectively are given in column 6. Smaller numbers than usual were employed owing to the relative difficulty of giving intravenous injections of chloral hydrate quickly. The numerals given in brackets in cols. 4 and 5 are the standard deviations of the mean sleeping times.

Drug (1)	Chloral Hydrate Dose (mg./kg.) (2)	Chloral Hydrate Route (3)	Sleeping Time (min.) Treated Group (4)	Sleeping Time (min.) Control Group (5)	No. in Group (6)
5-HT	112·5	i.v.	66 (26)	21 (7·0)	4/4
,, ...	225	i.p.	70 (18)	29 (11)	9/9
Methoxamine ..	75	i.v.	20 (6·5)	8 (7·1)	7/7
,, ...	200	,,	64 (20)	22 (9·5)	5/5
,, ...	225	i.p.	66 (14)	18 (5·3)	10/10
Tolazoline ..	75	i.v.	8 (8)	8 (7)	7/7
,, ..	200	,,	46 (14)	22 (9·5)	5/5
,, ..	225	i.p.	46 (17)	18 (5·2)	10/10

The antidiuretic activity of a compound such as 5-HT might prolong the effects of certain hypnotic drugs by delaying their excretion. Experiments were therefore performed to see whether 5-HT could prolong the effects of drugs which are mainly excreted in the urine.

Hexamethonium appeared to be a suitable drug for the purpose because it is entirely excreted in the urine (Paton and Zaimis, 1952) and its mydriatic effect can be readily measured in the mouse. Fig. 1 shows that the mydriatic effect of hexamethonium in mice is much prolonged by 5-HT. The latter alone caused a slight, transient mydriasis in mice. This mydriatic effect may have been brought about indirectly, for it was observed that 5-HT slowed down the accommodation to the light of the lamp used for constant illumination in measuring pupil size. In mice treated with 5-HT the light reflex resulted in pupillary constriction when the mice were brought near the lamp only after some 10 to 45 sec., compared with 5 to 10 sec. for normal mice. Thus, the rapid measuring of pupil size could lead to the recording of a spurious mydriasis.

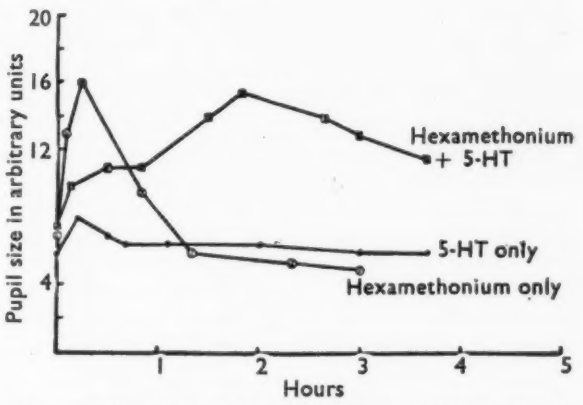

FIG. 1.—Prolongation of hexamethonium mydriasis in mice by premedication with 5-HT. Each point is the mean of 10 observations. The hexamethonium bromide (20 mg./kg. i.p.) was given 10 min. after 5-HT (20 mg./kg. s.c.). The same doses were used in control experiments with each compound alone.

Analogous experiments were performed with 5-HT and Intocostrin, which is largely excreted in the urine (Marsh, 1952; Kalow, 1953). In this case, the proportion of mice dying from respiratory failure after substantial doses of Intocostrin was taken as a measure of activity. The proportion was increased by treatment with 20 mg./kg. of 5-HT (Table IV).

TABLE IV

EFFECT OF 5-HT ON THE TOXICITY OF INTOCOSTRIN

The Intocostrin dose was given in standard rabbit head drop units and the drug was administered intraperitoneally 10 min. after the 5-HT (20 mg./kg. s.c.).

Intocostrin Dose	Survivals Treated Group	Survivals Control Group	Deaths Treated Group	Deaths Control Group
4·4 units	0	4	10	6
3·3 ,, ...	1	8	9	2
2·6 ,, ...	4	10	6	0

Influence of Lowered Body Temperature.—In the preceding experiments, mice pre-treated with 5-HT always felt colder than the controls. On one cold winter day, as the room temperature dropped, the mice which had received 5-HT became more and more flaccid instead of showing signs of waking from the chloral hydrate sleep. Heat was then applied by bringing two 75-watt lamps near the mice. Within 10 min. of the application of heat the mice began to wake up. This observation suggested that 5-HT might interfere with the temperature-regulating mechanism of the mice and prolong hypnotic action by lowering body temperature.

An experiment was then performed in which two groups of mice, one of which had received

5-HT (20 mg./kg. subcutaneously) and the other control injections of saline, were exposed to a cold environment. After both groups of mice had been kept in the refrigerator for 100 min. the average fall in the oral temperature of the treated mice was 5.8° C., whereas that for the control mice was 1.2° C. A t test showed the difference between the two sets of temperature readings to be highly significant ($t_s = 8.4$). Measurement of the rectal temperature also showed a highly significant ($t_s = 9.3$) difference. Whereas the mean rectal temperature for the treated mice fell from 38.3° C. (s.e. 0.7° C.) to 28.4° C. (s.e. 2.0), a difference of 9.9° C., the mean rectal temperature for the control group fell from 38.7° C. (s.e. 0.4) to 35.5° C. (s.e. 0.7), a difference of only 3.2° C.

Next, the rectal temperatures in groups of 5 mice which had been treated respectively as shown below were compared. Group I were given a subcutaneous injection of saline followed 10 min. later by an intraperitoneal injection of chloral hydrate (200 mg./kg.). Group II received a subcutaneous injection of 5-HT (20 mg./kg.) followed 10 min. later by an intraperitoneal injection of chloral hydrate (200 mg./kg.). Group III were injected subcutaneously with 5-HT (20 mg./kg.) followed 10 min. later by an intraperitoneal injection of saline. The results obtained are shown in Fig. 2. The difference in the average drop in

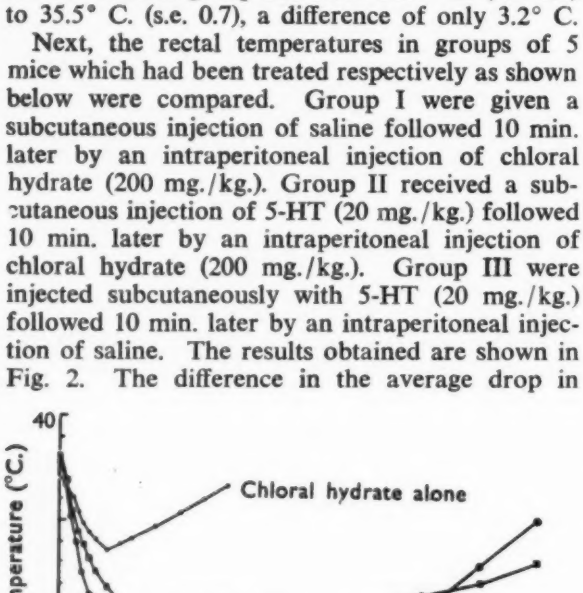

FIG. 2.—Effect of chloral hydrate and 5-HT on the rectal temperature of mice. Each point is the mean of observations made on 5 mice. Chloral hydrate (225 mg./kg. i.p.) and 5-HT (20 mg./kg. s.c.) were given either singly or in combination, in which case 5-HT was given 10 min. before chloral hydrate.

rectal temperature for the mice in Groups I and II respectively was highly significant both in extent and duration. Whereas the maximum temperature drop in Group II was 10.0° C., that in Group I was only 4.8° C.

In another series of experiments, some of the mice were kept at a temperature of 38° C. to prevent them losing heat to the surroundings, while others were kept at room temperature (20° C.). Four groups, each of 10 mice, were used. Two groups were given 5-HT (20 mg./kg.) followed by an intraperitoneal injection of chloral hydrate (225 mg./kg.). One of these groups was kept at 38° C. and the other at 20° C. The other two groups were treated similarly except that they received a control injection of saline instead of 5-HT. The average sleeping times of the four groups were as follows: 37.5 min. (s.e. 7 min.) for the mice which had received 5-HT and chloral hydrate and were kept at 38° C.; 82.5 min. (s.e. 2 min.) for the mice which had received 5-HT and chloral hydrate and were kept at 20° C.; 15 min. (s.e. 2 min.) for the mice which had received only saline before the chloral hydrate and which were kept at 38° C.; 17 min. (s.e. 3.5 min.) for the mice which had received only saline before the chloral hydrate and which were kept at 20° C.

These results show that keeping the mice at body temperature markedly reduces the prolonging effect of 5-HT on the chloral hydrate sleeping time. The difference between the average sleeping times for the first and third groups was highly significant statistically ($P<0.001$), as was the difference between the average sleeping times for the second and fourth group. The difference between the average sleeping times for the first two groups was significant at the $P<0.05$ level. Thus it seems that the hypothermic action of 5-HT may contribute largely to its hypnosis-prolonging activity.

Fig. 3 illustrates the results of an experiment in which four groups, each of 5 mice, received respectively: (*a*) adrenaline (5 mg./kg. subcutaneously) followed 10 min. later by chloral hydrate

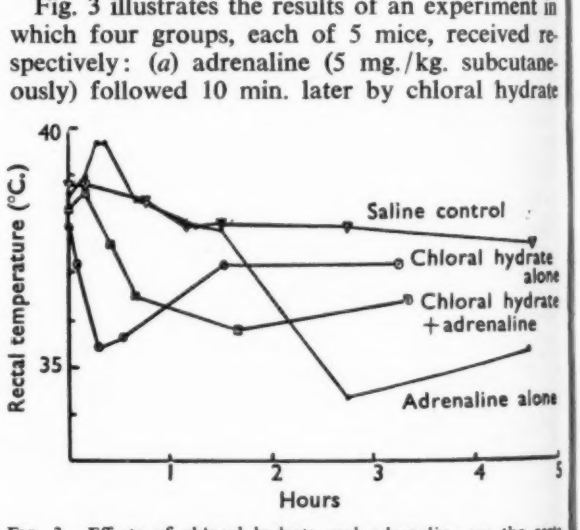

FIG. 3.—Effects of chloral hydrate and adrenaline on the rectal temperature of mice. Each point is the mean of 10 observations. Chloral hydrate (250 mg./kg. i.p.) and adrenaline (5 mg./kg. s.c.) were given either singly with control injections of saline or in combination, in which case the adrenaline was given 10 min. before the chloral hydrate.

(250 mg./kg. intraperitoneally); (*b*) a control injection of saline followed 10 min. later by the same dose of chloral hydrate; (*c*) adrenaline (5 mg./kg. subcutaneously) followed 10 min. later by a control injection of saline intraperitoneally; (*d*) a control injection of saline subcutaneously followed 10 min. later by another control injection intraperitoneally. It can be seen that adrenaline alone at first increased rectal temperature by 1° C. but later caused it to drop by 4° C. Chloral hydrate alone produced a sharp, substantial, but temporary fall of rectal temperature of 2.8° C. When adrenaline was given before chloral hydrate, however, there was a sustained fall of body temperature.

Discussion

The results obtained with sympathomimetic amines (Table I) suggested that there might be a connexion between vasoconstrictor activity and hypnosis-prolonging activity. However, the fact that such drugs as yohimbine, tolazoline, histamine, physostigmine, and atropine can also increase chloral hydrate sleeping time under comparable conditions (Table II) forces us to conclude either that (*a*) several quite distinct mechanisms of action are involved, or (*b*) the mechanism of action, if there be only one, is not directly related to the circulatory activity of the compounds.

Although the compounds known to possess hypnotic-potentiating activity differ greatly in their chief effects on the circulatory system, there is one property which the majority of them possess and which could explain their ability to potentiate the effects of hypnotics—namely, the ability to lower body temperature, especially in the presence of an hypnotic. Figs. 2 and 3 show the magnitude of the changes in body temperatures produced by doses of 5-HT and adrenaline which substantially increased the chloral hydrate sleeping time. These observations naturally raised the question, Do the other drugs which prolong chloral hydrate sleeping time have a similar effect on body temperature?

Numerous reports in the literature indicate that most of the compounds shown to have hypnotic-prolonging activity (Tables I and II) can lower body temperature to a noteworthy extent—for example, histamine (Fabinyi-Szebehely and Szebehely, 1952; Kind, 1954), ergot alkaloids (Githens, 1917; Flacke *et al.*, 1953), yohimbine (Dimitrijević, 1938), tolazoline (Horwitz, Montgomery, Longaker and Sayen, 1949), and atropine (Burn and Dutta, 1948).

As many investigators have reported that adrenaline has a calorigenic action, resulting in an increase in body temperature and in oxygen consumption, we thought it important to see how adrenaline affected the body temperature of mice under the experimental conditions of this study. The results illustrated in Fig. 3 show that mice pre-treated with adrenaline have a much more prolonged fall in body temperature when given chloral hydrate than control mice. Gyermek (1950) found in rats that the effect of adrenaline on oxygen consumption and body temperature depended on the environmental temperature: at 20° C., adrenaline (0.5 mg./kg. s.c.) decreased both oxygen consumption and body temperature, whereas at 30° C. it increased them. From such findings, many of which have been reviewed by Griffith (1951), it appears that the variety of effects which adrenaline has been reported to produce (according to the dose, animal species, and other experimental conditions) can be explained best by assuming that adrenaline can affect body temperature by several distinct actions which may be opposed. Thus an action on the central nervous system tending to lower body temperature may be opposed by a peripheral action, such as the liberation of glucose, which tends to raise it.

Some of the drugs (colchicine, certain "tranquillizers," and antihistamines) which have been found by other investigators to prolong the effects of hypnotic drugs have also been shown to have a hypothermic action. When Burn and Dutta (1948) observed that procaine shares its body temperature lowering action in mice with such drugs as atropine, diphenhydramine, pethidine, and quinidine, all of which produce a number of pharmacological effects attributable to antagonism of particular actions of acetylcholine, they suggested "that the maintenance of body temperature depends on a mechanism in which acetylcholine plays a part." This does not necessarily imply that the action is a central one. However, it seems likely that the chief site of the hypothermic action of compounds like atropine and quinidine is in the central nervous system, possibly in the hypothalamus.

Even if many of the compounds which prolong the effects of hypnotics do lower body temperature under comparable conditions, there may be no causal relationship between the two effects; both effects might be the result of an action affecting chiefly the hypothalamic region of the brain. Against this interpretation is the fact that some of the compounds which prolonged chloral hydrate sleeping time are ones like histamine,

whose effects on body temperature are likely to be brought about principally by a direct action on peripheral blood vessels. Moreover, it has been shown in the case of 5-HT that the sleep-prolonging effect is much reduced when the environmental temperature is raised to make heat loss minimal.

One finding which was disconcerting at first was the great variation in the mean sleeping times for the control groups of mice. We now attribute this mainly to the fact that the laboratory temperature varied considerably from day to day. The importance of temperature control was realized subsequently. Because of the variation in mean sleeping times, the effect of a compound on sleeping time was usually tested more than once and by different observers. The results of duplicated experiments were usually concordant.

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References

Blackman, J. G., Fastier, F. N., Patel, C. M., and Wong, L. C. K. (1956). *Brit. J. Pharmacol.*, **11**, 282.

Burn, J. H., and Dutta, N. K. (1948). *Nature, Lond.*, **161**, 18.

Dimitrijević, I. N. (1938). *Prwi Rasred.*, **177**, 247. Cited in *Chem. Abstr.*, **34**, 7414.

Fabinyi-Szebehely, M., and Szebehely, J. (1952). *Acta physiol. scand.*, **27**, 1.

Fastier, F. N. (1956). *Experientia*, **12**, 351.

Flacke, W., Mülcke, G., and Schulz, R. (1953). *Arch. exp. Path. Pharmak.*, **220**, 469.

Fornaroli, P., and Koller, M. (1955). *Farmaco*, **9**, 546.

Fuhrman, F. A. (1946). *Physiol. Rev.*, **26**, 247.

Githens, T. S. (1917). *J. Pharmacol.*, **10**, 327.

Griffith, F. R. (1951). *Physiol. Rev.*, **31**, 151.

Gyermek, L. (1950). *Arch. exp. Path. Pharmak.*, **209**, 456.

Horwitz, O., Montgomery, H., Longaker, E. D., and Sayen, A. (1949). *Amer. J. med. Sci.*, **218**, 669.

Kalow, W. (1953). *J. Pharmacol.*, **109**, 74.

Kind, L. S. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 371.

Marsh, D. F. (1952). *J. Pharmacol.*, **105**, 299.

Paton, W. D. M., and Zaimis, E. J. (1952). *Pharmacol. Rev.*, **4**, 219.

Richards, R. K., and Taylor, J. D. (1956). *Anesthesiology*, **17**, 414.

Salmoiraghi, G. C., Sollero, L., and Page, I. H. (1956). *J. Pharmacol.*, **117**, 166.

Shore, P. A., Silver, S. L., and Brodie, B. B. (1955). *Experientia*, **11**, 272.

Werle, E., and Lentzen, J. (1938). *Arch. exp. Path. Pharmak.*, **190**, 328.

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THE EFFECT OF PHENYLBUTAZONE UPON DIHYDROTACHYSTEROL OVERDOSAGE IN THE RAT

BY

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(RECEIVED MARCH 19, 1957)

Experiments on albino rats showed that short-term overdosage with dihydrotachysterol, in amounts causing only traces of soft-tissue calcification, induced a very pronounced calcium deposition in the heart, aorta, and kidney when the animals were simultaneously treated with phenylbutazone. Special attention is called to the fact that, under these experimental conditions, it is possible to obtain consistently a massive calcium deposition in the stroma of the renal papilla similar to that seen in " Randall's plaques " during the development of urolithiasis in man.

It had been shown in earlier publications that corticoid hormones can significantly modify the syndrome of soft-tissue calcification that is induced by vitamin-D derivatives (Selye, 1956, 1957). It seemed of interest to establish whether this singular effect of the adrenocortical hormones could be duplicated with phenylbutazone, since many of the pharmacological actions of the latter resemble those of corticoids.

METHODS

Forty female Sprague-Dawley rats with a mean initial body weight of 101 g. (range, 92 to 110 g.) were subdivided into four equal groups. Treatment with dihydrotachysterol and phenylbutazone was carried out as is shown in Table I. Dihydrotachysterol (" Calcamin," Wander) was given once daily in doses of 100 μg. in 0.4 ml. of sesame oil through a stomach tube. Phenylbutazone (" Butazolidin," Geigy) was administered subcutaneously in two daily injections of 10 or 20 mg. each in 0.2 ml. of water.

Throughout the experiment the animals were fed on " Purina Fox Chow " (Ralston Purina Company Ltd.). After 10 days' treatment all the surviving rats were killed with chloroform ; their hearts, aortae, and kidneys were fixed in neutral formalin, embedded in paraffin, and stained with von Kóssa's calcium stain. The intensity of calcification was assessed in an arbitrary scale of 0 to +++ (0=no calcification, + = trace of calcification, ++=moderate degree of calcification, +++=intense calcification). The mean of these values as well as the mean final body weight (with its standard error), the % gain in body weight during the period of observation, and the mortality rate are summarized in Table I.

RESULTS

During the last 3 days of the experiment, one rat in Group III and four rats in Group IV died with extensive calcium depositions in the heart, aorta and kidney. It was particularly striking that the renal calcification remained almost exclusively limited to the papilla. It will be remembered that the rat kidney possesses only one papilla.

When survivors were killed on the 10th day, it became evident that, in all animals treated with dihydrotachysterol in combination with phenylbutazone, the renal papillae were chalky white in

TABLE I

EFFECT OF PHENYLBUTAZONE UPON DIHYDROTACHYSTEROL OVERDOSAGE IN THE RAT

Each group consisted of 10 rats. The arbitrary scale of assessment of the degree of tissue calcification is given in the text.

Group	Phenylbutazone (mg./day)	Dihydrotachysterol (μg./day)	Final Body Weight with S.E. (g.)	Gain in Body Weight (%)	Calcification			Mortality (%)
					Heart	Aorta	Kidney	
I	—	100	83±1·5	−18	0	0 to +	0 to +	0
II	2×20	—	128±4·3	+28	0	0	0	0
III	2×10	100	77±2·2	−23	+ to ++	+++	+++	10
IV	2×20	100	73±2·9	−26	++	+++	+++	40

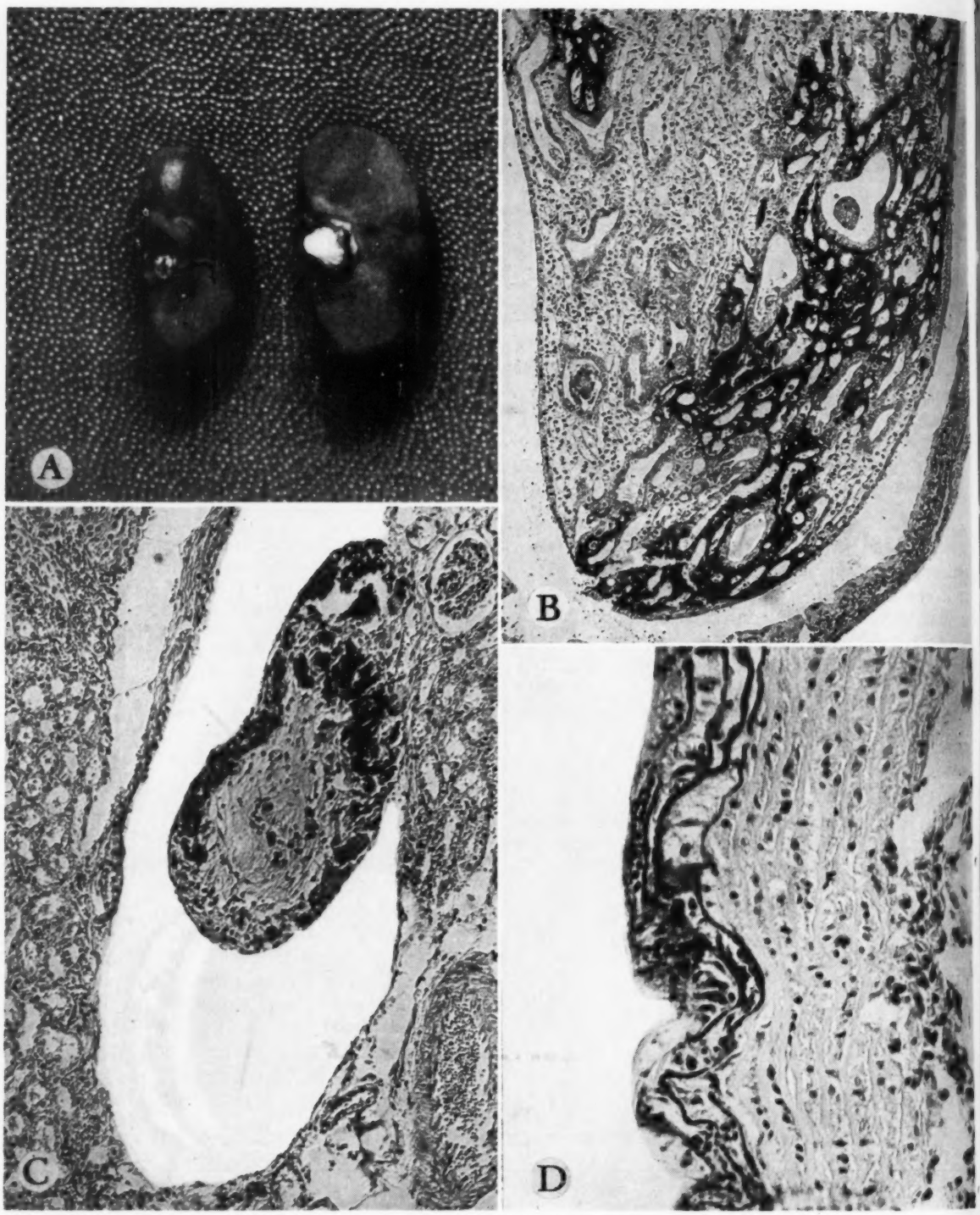

FIG. 1.—A. Kidney of a rat treated with dihydrotachysterol alone (left) and of an animal given dihydrotachysterol and phenylbutazone. Note the calcification (chalky white colour) and great prominence of the renal papilla in the latter kidney. (The light-grey areas in both kidneys are reflections of light unrelated to calcification.) B. Histological aspect of the renal papilla in a rat treated with dihydrotachysterol plus phenylbutazone. The localization of the calcium deposits in the stroma is clearly visible here with von Kóssa stain. ×75. C. Partially calcified "venous cushion," which protrudes from the wall into the lumen of one of the large renal veins near the hilum of the rat kidney shown in B, von Kóssa stain. ×75. D. Wall of the aorta showing the Mönckeberg sclerosis-like calcification in a rat treated with dihydrotachysterol and phenylbutazone, von Kóssa stain. ×320.

colour, and histochemical examination with von Kóssa's stain revealed that this region was deeply impregnated with calcium salts. Calcification was restricted almost wholly to the stroma between the collecting ducts. There was no tendency to form calcified casts such as are observed, for example, in rats which have received a great excess of sodium phosphate in the diet. This incrustation of the renal papillary stroma with calcium is strikingly similar to the initial stages of renal calculus formation, the so-called " Randall's plaque " (Randall, 1937). In two of the animals in Group IV, hard calcified calculi were found loose in the renal pelvis. These stones were presumably caused by the detachment of the calcified area from the papilla and the subsequent deposition of salts upon these foci ; all this is in agreement with Randall's theory.

It is also noteworthy that in Group IV polyp-like protuberances frequently developed in the larger renal veins. These are apparently identical with the " venous cushions " seen in rats simultaneously treated with corticoids and ergocalciferol (Selye, 1956). However, the " venous cushions " in Group IV of the present experiment were invariably calcified whereas those of the rats treated with corticoids and ergocalciferol were not incrustated with calcium salts.

Intense calcification was seen in the aortae and hearts of the rats in Groups III and IV which received phenylbutazone simultaneously with dihydrotachysterol. On the other hand, no calcification was observed in these organs after treatment with phenylbutazone alone, and only traces of calcium were detectable in the aortae and kidneys of the rats treated with dihydrotachysterol alone, in these short-term experiments.

The loss in body weight induced by dihydrotachysterol alone was significantly increased by twice-daily doses of 20 mg. of phenylbutazone. On the other hand, phenylbutazone alone (given at this same dosage) not only failed to cause a loss in body weight but actually permitted a gain of 28% in 10 days, which is approximately normal for rats of this weight range in our colony.

These findings are summarized in Table I and illustrated in Fig. 1.

Discussion

From these observations it is clear that the syndrome of dihydrotachysterol overdosage is significantly modified by concurrent treatment with phenylbutazone. The loss of body weight and the tendency to form calcium deposits in heart, aorta and kidney, during a brief period of treatment with dihydrotachysterol, was greatly enhanced by the concurrent administration of, in themselves, non-toxic doses of phenylbutazone. In all these respects phenylbutazone altered the syndrome of dihydrotachysterol intoxication in much the same way as hydrocortisone acetate acts upon the syndrome of intoxication produced by ergocalciferol in the rat (Selye, 1956). However, combined treatment with hydrocortisone acetate and ergocalciferol results in calcifications, localized predominantly in the aorta, whilst calcification in the renal tissue is less conspicuous and usually limited to the formation of calcified casts (especially in the cortex and the corticomedullary junction line). Combined treatment with phenylbutazone and dihydrotachysterol produces calcium deposition predominantly in the stroma of the renal papillae.

The evidence at our disposal does not warrant the formulation of any hypothesis concerning the mechanism of the interaction between phenylbutazone and dihydrotachysterol. However, it does show that combined treatment with these two substances regularly resulted in the production of changes similar to those in " Randall's plaques " in the lower kidney.

These investigations were carried out with the aid of a grant from Geigy Pharmaceuticals. The author is also indebted to Wander Pharmaceutical Products for the dihydrotachysterol (" Calcamin "); to Geigy Pharmaceuticals for the phenylbutazone (" Butazolidin ") used in these experiments; and to Mr. Kai Nielsen for the preparation of the photographs.

References

Randall, A. (1937). *Ann. Surg.*, **105**, 1009.
Selye, H. (1956). *Münch. med. Wschr.*, **31**, 1015.
—— (1957). *Acta Endocr., Copenhagen.* In the press.

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THE INTERACTION BETWEEN EDROPHONIUM (TENSILON) AND ACETYLCHOLINE AT THE MOTOR END-PLATE

BY

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The effect of edrophonium (3-hydroxy-phenyl-dimethylethylammonium chloride) on the motor end-plate and its interaction with acetylcholine and carbachol has been investigated. Use was made of intracellular recording of membrane potential and of ionophoretic micro-application of drugs from single and twin-pipettes.

Small doses of edrophonium potentiate the depolarizing effect of acetylcholine, but not that of carbachol. This action can be observed with doses of edrophonium which have no depolarizing effect by themselves. Large doses of edrophonium have some depolarizing action and, at the same time, inhibit depolarizations produced by carbachol. After treatment with neostigmine, edrophonium fails to potentiate the acetylcholine response. The observations are in agreement with the view that the principal action of edrophonium on the neuromuscular junction is that of a potent and rapidly acting anticholinesterase.

The effects of anticholinesterases on muscle are usually tested under conditions in which the inhibitor/enzyme reaction has approached or reached equilibrium. In recent experiments (Castillo and Katz, 1957c), a different method was used, brief localized doses of the drug being applied to an end-plate with the help of an ionophoretic micro-technique. Under these conditions, the observed potency of a drug depends on the kinetics, rather than the equilibrium constant, of the reaction. In such experiments it was found that substances like neostigmine, which are strong but slowly acting enzyme inhibitors, produced no potentiation of the acetylcholine response, while less powerful but more rapidly acting esterase inhibitors (choline, decamethonium) caused a marked increase in the acetylcholine effect.

Similar experiments will be described in which edrophonium (3-hydroxy-phenyl-dimethylethylammonium chloride) was allowed to interact with acetylcholine (ACh), by applying the substances from micropipettes placed at close range to an end-plate of the frog's sartorius muscle. The membrane potential of the muscle fibre was recorded with an intracellular electrode inserted within a few hundred microns of the point of drug action.

The effect of edrophonium on neuromuscular transmission in the frog has previously been studied by Nastuk and Alexander (1954), who concluded that the anticurare action of this substance and the modifications which it produced in the shape of the electric end-plate response could be attributed to its anti-esterase activity (see also Smith, Cohen, Pelikan, and Unna, 1952). The present experiments confirm this view and provide additional evidence for the high speed at which the reaction between edrophonium and ACh-esterase proceeds.

METHOD

The technique has been described in detail in previous papers (Castillo and Katz, 1955, 1957a, c; see also Katz and Thesleff, 1957). The experiments were made on isolated sartorius muscles of *R. temporaria* at about 20° C. The preparations were mounted in a bath of Ringer solution which contained the electrodes for the recording of membrane potentials and for the electrophoretic application of drugs. Single or twin-pipettes were used, containing edrophonium and ACh, or edrophonium and carbachol, in the twin barrels. Edrophonium (Tensilon) was obtained by courtesy of Roche Products. Individual drug pipettes had tip diameters of less than 1 μ and were filled with a concentrated solution (0.5 to 2.5 M). The discharge of the drug was regulated by "braking" or "releasing" voltages (making the interior of the pipette more negative, or positive, respectively), in the way described in the earlier papers (Castillo and Katz, 1955, 1957a). The current flowing through the drug pipettes was registered on the second beam of the oscilloscope.

RESULTS

Fig. 1 illustrates the potentiating action of edrophonium. The records show potential changes produced at the end-plate region of a muscle fibre when ACh and edrophonium were discharged from a twin pipette in the immediate neighbourhood. In this experiment ACh was released from one barrel by slightly reducing the steady "braking" current which passed through it. This gave rise to a steady depolarization causing an upward displacement of the baseline from *a* to *b*, and then to *c*. The bottom trace serves to register the current through the pipettes, but the changes in the ACh-pipette were so small (of the order of 10^{-9} A.) that no visible displacement in the three successive lines occurred. The brief deflexion which interrupts this trace arose from a pulse through the edrophonium pipette (about 1.4×10^{-8} A., duration 13 msec.). The discharge had practically no effect in record *a* when ACh efflux was prevented. In record *b*, when a small efflux of ACh was present producing a steady depolarization of about 0.5 mV, this potential change increased to 3 mV after the edrophonium pulse. In record *c*, a steady ACh potential of 4 mV was increased to 14 mV by the same pulse of edrophonium.

It appeared from these results that a momentary application of edrophonium which, by itself, produced no potential change (record *a*), caused a several-fold increase of the depolarizing effect of ACh. The time course of this potentiation was rapid and had practically subsided within less than one second.

The effect shown in Fig. 1 might be explained by a rapid and quickly reversible anti-esterase action of edrophonium. To test this assumption,

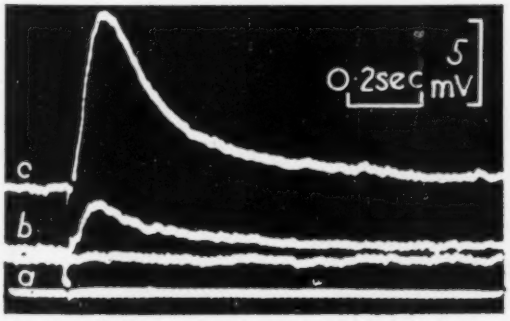

FIG. 1.—Potentiation of steady ACh-potential by a pulse of edrophonium. The pulse is shown in the bottom trace. It had no effect in (*a*) (when ACh-efflux was stopped by a small braking current), but produced a transient large increase of the steady depolarization in (*b*) and (*c*) (when a controlled small efflux from the ACh pipette occurred). See text for further details. The 5 mV scale refers to the membrane potentials (*a* to *c*). Calibration of the "current-monitor" (bottom trace): 5 mV scale $= 9.1 \times 10^{-8}$ A.

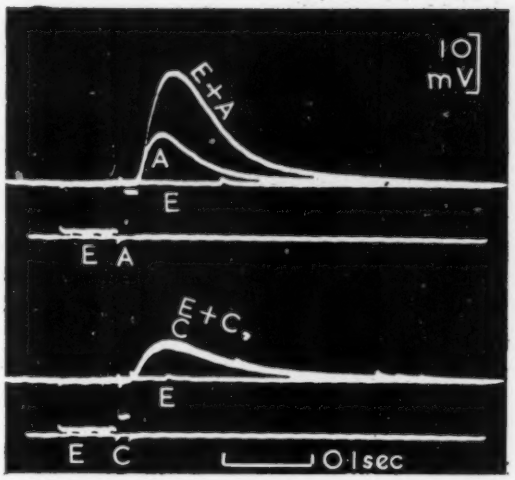

FIG. 2.—Upper part shows potentiation of a brief ACh-potential by a preceding edrophonium pulse. Lower part shows the barely noticeable effect produced by edrophonium on a carbachol potential. In each record, three traces were superimposed. E, edrophonium; A, acetylcholine; C, carbachol. See text for further details. Monitor calibration, 10 mV scale $= 6.7 \times 10^{-8}$ A.

two kinds of experiments were made: (*a*) effects of edrophonium were examined when ACh was replaced by a stable depolarizing substance (carbachol); (*b*) the interactions were studied before and after the muscle had been treated with neostigmine.

All the subsequent experiments were made with an assembly of three drug-pipettes, one—a twin pipette—containing edrophonium and carbachol in the two barrels, another separate pipette containing ACh and being placed nearby. This arrangement was chosen to obviate the possibility that any increase in the ACh effect could have been brought about by leakage between the twin barrels. It should be noted that with this set-up, because of the closer proximity of edrophonium and carbachol pipettes, any effect which edrophonium may have on the carbachol response would be more easily detected than interactions between edrophonium and ACh.

In Fig. 2, an example of the results is shown. In the upper part, three records are superimposed in which an edrophonium pulse (E), an ACh pulse (A), and both pulses together (E + A), were applied. The edrophonium pulse by itself produced a minute depolarization, barely rising above the base-line. The ACh dose alone produced the smaller of the two main deflexions. When the ACh pulse was preceded by the edrophonium pulse, the amplitude of the deflexion was more than doubled. In the lower part of the figure a similar series of three records is superimposed, but this time carbachol was used instead of ACh.

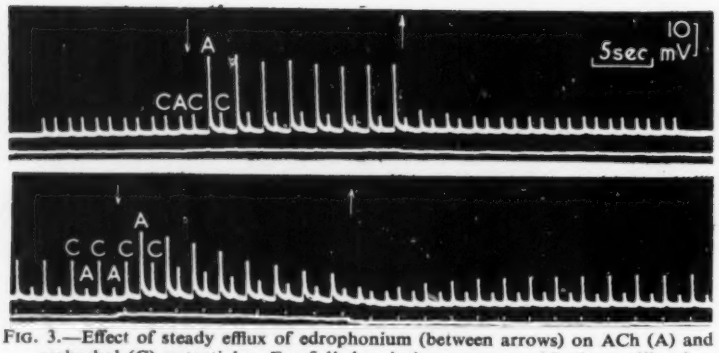

FIG. 3.—Effect of steady efflux of edrophonium (between arrows) on ACh (A) and carbachol (C) potentials. For full description, see text. Monitor calibration, 10 mV scale $=8\times10^{-8}$ A.

The combination of edrophonium + carbachol pulses produced only a very slightly increased effect above that due to the carbachol pulse alone.

That the edrophonium-potentiation is observed specifically with ACh, and not with carbachol, is brought out in a somewhat different fashion in Fig. 3. Here, alternate pulses of ACh and carbachol were applied to the end-plate, and the resulting brief depolarization recorded on slowly moving film. During the interval marked by arrows, the "brake" on the edrophonium barrel was reduced or reversed (signalled by a very small upward displacement of the bottom trace), and a steady efflux of this substance, therefore, occurred. In the upper part of Fig. 3, the pulses of ACh and carbachol had been adjusted initially so that the responses were of approximately the same amplitude. As soon as edrophonium began to be released, a large increase of the ACh-potentials occurred, while the carbachol potentials remained practically unaltered. After the end of the edrophonium application, the ACh-potentials declined within a few seconds to the level of the carbachol responses.

In the lower part of Fig. 3, different dosages were chosen. The pulses had been adjusted so that initially the carbachol potentials were about three times larger than the ACh potentials. The steady edrophonium dose was also increased, to a strength at which it produced a small, but noticeable, steady depolarization. The effect of edrophonium was two-fold. The ACh response was potentiated, its amplitude now exceeding the carbachol potential. The initial increase, however, was not maintained, but the responses gradually declined during the edrophonium period. This decline affected ACh and carbachol potentials to the same extent, and the ACh potentials remained larger than the carbachol until the efflux of edrophonium was stopped. It is clear, therefore, that the decline was not due to a gradual diminution of the specific ACh potentiation by the drug, but to a progressive "desensitization" of receptors which occurs whenever a depolarizing substance is applied for a prolonged period (see Katz and Thesleff, 1957, for a detailed study of this phenomenon).

It can be concluded from these results that small doses of edrophonium, which by themselves do not appreciably alter the membrane p.d., increase the depolarizing effect of ACh, but not that of carbachol. This supports the view that the effect is brought about by inhibition of ACh-esterase.

When the dose of edrophonium was increased, the potentiation became intensified as shown in Fig. 4 (left part). The method was the same as employed in Fig. 1: an edrophonium pulse was applied during a period of steady ACh depolarization. The bottom traces in each frame show an

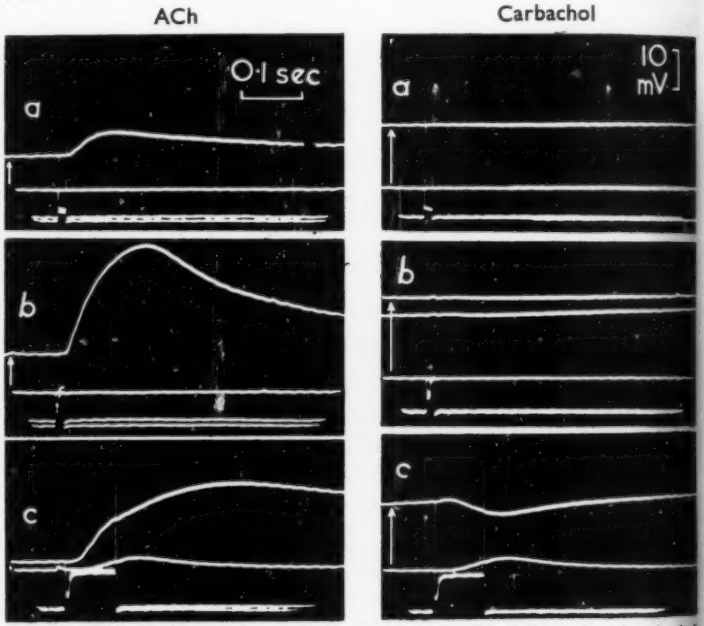

FIG. 4.—Effect of different doses of edrophonium on ACh (left part) and carbachol potentials (right part). The procedure was the same as in Fig. 1, a steady dose of ACh or carbachol being combined with a pulse of edrophonium. The depolarization due to ACh or carbachol is shown by the displacement of the trace in the direction of the arrow. The records in each horizontal row were obtained with the same edrophonium dose, whose strength was increased from *a* to *c*. Note that in *a* and *b*, the edrophonium by itself had no effect on the membrane potential, while it produced a small, transient depolarization in *c*. For further description, see text. Monitor calibration, 10 mV scale $=7.9\times10^{-8}$ A.

edrophonium pulse, whose coulomb strength was increased from *a* to *c*. In each frame, two records are shown, (1) when ACh efflux has been stopped by a large " braking " current, and (2) when ACh release was allowed to occur. In frame *c*, a very small steady dose of ACh was given, producing only 2 to 3 mV. depolarization ; this was a necessary precaution to avoid excitation and twitching. Potentiation increased, from a factor of 1.7 in *a*, to about 10 times in *c*. In addition, the effect was lengthened considerably.

It will be observed that in *a* and *b*, the edrophonium pulse by itself produced no potential change, while in *c* a transient depolarization of a few mV. was seen. This indicates that there is a substantial margin between the doses of edrophonium which interfere with ACh esterase, and those which combine effectively with the receptors (see also Nastuk and Alexander, 1954). In this respect, edrophonium may be classified as a " specific " anti-esterase, like neostigmine, but unlike decamethonium or choline which have a mixed action on esterase and receptors in the same dosage range (Castillo and Katz, 1957c).

The right-hand part of Fig. 4 shows, for comparison, interactions between edrophonium and carbachol at the same end-plate spot. Each horizontal row of records in Fig. 4 was obtained with identical edrophonium pulses. In the upper right frame, no effect is observed. In the middle frame, there is a barely noticeable trace of inhibition ; in the lower frame, the inhibitory effect of edrophonium on the carbachol-potential is well marked. We are dealing here with an example of competitive interference between two depolarizing drugs, of the kind described in detail by Castillo and Katz (1957c ; see also Ariëns, 1954 ; Stephenson, 1956).

Finally, the observations were repeated after the preparation had been treated for about 30 min. with neostigmine. With a concentration of 10^{-6} w/v (neostigmine methylsulphate/Ringer), the potentiating action of edrophonium was greatly reduced. With a two to four times larger dose of neostigmine, the potentiation of the ACh effect had vanished, and the interaction between edrophonium and ACh was now very similar to that observed with edrophonium and carbachol, that is, when relatively large doses were used, edrophonium now *inhibited* ACh as well as carbachol potentials. An example is shown in Fig. 5. The upper records show the depolarization produced by the large edrophonium pulse. The middle frames show an ACh and a carbachol potential, respectively ; the lower frames show the depression of both ACh and carbachol effects when they are preceded by an edrophonium pulse.

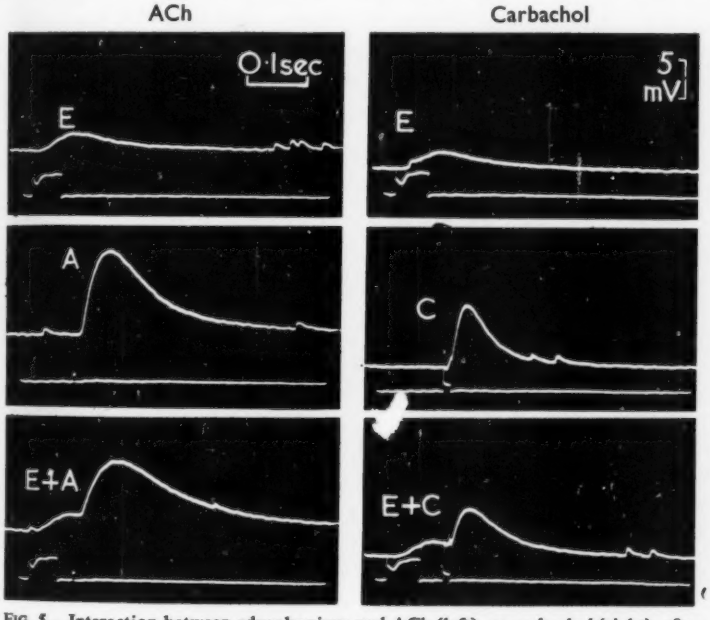

FIG. 5.—Interaction between edrophonium and ACh (left), or carbachol (right), after pre-treatment with neostigmine. The procedure was similar to that in Fig. 2, except that a large dose of edrophonium was used which depolarized by itself. E, edrophonium; A, ACh; C, carbachol. Note that the potentiation of the ACh effect (Fig. 4*c*, left) has now given place to inhibition, similar to that observed with carbachol. Monitor calibration, 5 mV scale=7.9×10^{-8} A.

Thus, initial treatment with a more slowly-acting, but powerful, anti-esterase abolishes the potentiation, and all that is left is the relatively weak depolarizing action of edrophonium and the associated inhibitory effect due to its competition with more powerful depolarizing agents.

DISCUSSION

The results fully support the conclusions reached by Nastuk and Alexander (1954) and by Smith *et. al.* (1952), namely that the principal action of edrophonium on neuromuscular transmission is that of an anticholinesterase. Application of this drug potentiates the effect of ACh, but not of its stable analogue, carbachol ; and the potentiation is not seen when the esterase activity has already been inhibited by neostigmine.

The time course of the potentiating effect is rapid. For example, in the experiment illustrated in Fig. 1, the effect of an edrophonium pulse rose

to a peak in 85 msec. and fell to one half in about 140 msec. which was only four times slower than a small depolarization produced at the same spot by a similar pulse of ACh from the adjacent pipette. In comparing the time courses of these two effects, a factor of two should be allowed for the enzymatic removal of ACh (see Castillo and Katz, 1957b), and some part of the remaining difference is probably due to slower diffusion rather than to the reaction kinetics. In any case, the bond between edrophonium and esterase must be rapidly reversible, with a time constant of dissociation of less than 0.1 sec. at 20° C. This is very much faster than the dissociation rate of the neostigmine- or eserine-enzyme complex (see Easson and Stedman, 1936; Eccles, Katz, and Kuffler, 1942; Augustinsson and Nachmansohn, 1949; Goldstein, 1951), which have time constants of the order of several minutes and exert their effects too slowly to be usefully investigated with the present method.

Some comment is needed on the relatively small interaction between edrophonium and carbachol. The reduction of a carbachol depolarization by a large pulse of edrophonium can be explained on the asumption that both drugs combine with receptor molecules, but that the edrophonium receptor complex has less "depolarizing efficacy" (see Stephenson, 1956) than the carbachol receptor compound.

With weaker doses of edrophonium, however, a small increase of the carbachol potential was often observed (Fig. 2). This was seen usually when the edrophonium application by itself produced a just noticeable depolarizing effect. It is probable that this small, positive, interaction arises from the fact that two *weak* doses of any depolarizing drug produce a more than additive effect, their dose/response relation having an S-shaped, rather than a linear, start (Katz and Thesleff, 1957).

We are indebted to Mr. J. L. Parkinson for his unfailing help, and to the Nuffield Foundation for financial assistance. It is a pleasure also to thank Dr. F. Hobbiger for valuable discussion. One of us (S.T.) was in receipt of a Travel Grant from the Swedish Medical Research Council.

References

Ariëns, E. J. (1954). *Arch. int. Pharmacodyn.*, **99**, 32.
Augustinsson, K. B., and Nachmansohn, D. (1949). *J. biol. Chem.*, **179**, 543.
Castillo, J. del, and Katz, B. (1955). *J. Physiol.*, **128**, 157.
—— —— (1957a). *Proc. roy. Soc. B.*, **146**, 339.
—— —— (1957b). Ibid., **146**, 362.
—— —— (1957c). Ibid., **146**, 369.
Easson, L. H., and Stedman, E. (1936). Ibid., **121**, 142.
Eccles, J. C., Katz, B., and Kuffler, S. W. (1942). *J. Neurophysiol.*, **5**, 211.
Goldstein, A. (1951). *Arch. Biochem. Biophys.*, **34**, 169.
Katz, B., and Thesleff, S. (1957). *J. Physiol.*, **137**, in the press.
Nastuk, W. L., and Alexander, J. T. (1954). *J. Pharmacol.*, **111**, 302.
Smith, C. M., Cohen, H. L., Pelikan, E. W., and Unna, K. R. (1952). Ibid., **105**, 391.
Stephenson, R. P. (1956). *Brit. J. Pharmacol.*, **11**, 379.

ERRATA

(*a*) In the paper by D. J. Holdstock, A. P. Mathias, and M. Schachter in the June, 1957, issue (Vol. 12, No. 2), the following error appeared in the 4th line of the legend to Fig. 9, on page 156. The times of incubation should read $2\frac{1}{2}$ and 10 min. and not 2 and $2\frac{1}{2}$ min. respectively.

(*b*) In the paper by D. D. Bonnycastle, N. J. Giarman, and M. K. Paasonen in the June, 1957, issue (Vol. 12, No. 2), an error appeared in Table II on page 229. The 5-hydroxytryptamine contents of brain, spleen, and small intestine are recorded in ng./g. and not ng./kg. as printed.

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THE DISTRIBUTION OF ACETYLCHOLINE IN THE MALAYAN JACK-FRUIT PLANT, *ARTOCARPUS INTEGRA*

BY

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(RECEIVED OCTOBER 29, 1956)

The distribution of acetylcholine in the seeds and leaves of the Malayan Jack-fruit plant, *Artocarpus integra*, has been studied with the view to obtaining evidence for the site of its formation. The terminal growing leaves on the side branches had a very high concentration of acetylcholine (770 μg./g.), while the acetylcholine content of the other leaves on the same branch progressively decreased with age. The total amount of acetylcholine stored in the terminal growing leaves was only 42 μg., but in the second leaves which had grown nearly to their full size it was 540 μg. From the third leaves, the amount of acetylcholine stored gradually decreased. The midribs and the secondary veins of the leaves when combined had a higher concentration of acetylcholine than had the blades. The acetylcholine concentration of the pith of the stem was 4.2 times higher than that of the cortex-phloem layer while that of the xylem layer was the lowest; in the root the pith had a value only one-seventh of the cortex. The younger part of the pith and the cortex-phloem layers of the stem contained more acetylcholine than the older parts. These findings support the view that the acetylcholine is synthesized in the growing leaves. An unusual lenticel-like structure in the cortex layer of the root contained more acetylcholine than the surrounding tissue.

In a previous paper (Lin, 1955) it was reported that considerable amounts of acetylcholine (ACh) are present in the seeds and leaves of the Malayan Jack-fruit, *Artocarpus integra*, Merr. It was suggested that it would be of interest to find out in which part of the plant the ACh is formed. For locating the site of formation of other pharmacologically active substances in plants, a method of interspecific grafts involving members of different genera which have a different capacity for alkaloid production has been employed with some success by James (1953). For instance, James and Thewlis (1952) by studying grafts prepared from belladonna and *Datura innoxia* concluded that the root was the site of synthesis of the alkaloids hyoscine and hyoscyamine in these plants, and Evans and Partridge (1953) from a study of the distribution of alkaloids in grafts involving *Datura tatula* and *D. feroxia* found that the aerial parts of these plants were capable of synthesizing hysocyamine and meteloidine respectively. A similar method of approach could probably be employed for locating the site of formation of ACh in the Jack-fruit plant. However, since the concentration of ACh in the tissues is so high and its assay is possible even with as small an amount of material as 10 to 20 mg. of the pith of a stem or of the external cortex of the root, it was decided to measure the ACh concentration of representative portions of the whole plant.

METHODS

Only fresh tissues which had just been taken from the living Jack-fruit tree were used. The process of extraction of ACh from the various tissues of the plant and the procedure adopted for its identification were similar to those previously described (Lin, 1955). For the routine assay of ACh the isolated toad rectus abdominis muscle preparation was used. The values of ACh given refer to acetylcholine chloride.

RESULTS

Acetylcholine in the Leaves

Variation of ACh Concentration with Age.— The exact age of a leaf in a tree is not easy to determine unless its growth has been followed from the beginning. The relative age of the leaves grown on a side branch of a tree, however, is known to follow the order of the position of growth along the stem ; a lower leaf is older than

the one above it and the top leaf is the youngest leaf of all those on the same branch. On this assumption the ACh concentration of each of a number of leaves was studied in relation to their age. For each experiment, a young side-branch of a Jack-fruit tree having 10 or 11 leaves was chosen. Immediately after the branch was cut down from the tree and brought to the laboratory, the leaves were removed from the stem one at a time, starting from the youngest leaf on the top to the lowest one at the bottom of the branch in the order of their position. Each leaf was at once cut into small pieces and ground in a mortar with some sand and with frequent additions of HCl-acidified water until a thorough extraction of the ACh had been obtained. The extract was centrifuged and the supernatant fluid assayed for ACh.

Fig. 1 shows the average results of three such experiments. Leaf No. 1 was the terminal growing leaf, which was still surrounded by the stipules, whilst the others, except the second leaf, were older and fully grown leaves, the age of which increased with their numbers in the table. In all experiments, the terminal growing leaves had the highest concentration of ACh and that of the other leaves decreased with their age. Fig. 1 also shows the total amount of ACh contained in each leaf. It is seen that the average amount stored in the second leaves is about 13 times more than that in the terminal growing leaves. This high value for ACh content is, however, confined to the second leaves, for that in all older leaves decreases.

ACh Concentration in the Terminal Growing Leaves.—A number of the terminal growing leaves were taken, each from a separate twig. After weighing, each leaf was extracted as in the previous experiments. The weights of these leaves are taken to represent the relative age of their growth, the heavier leaves being older. The results obtained showed a very high concentration of ACh in these terminal growing leaves, varying from 500 to 1,200 μg./g., with an average value of 770 μg./g. from 14 leaves. No correlation, however, was found to exist between the ACh concentration of each leaf and its weight.

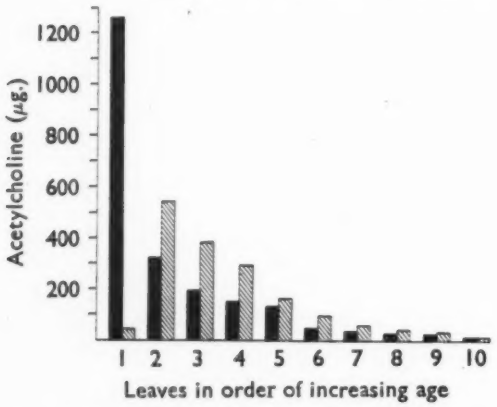

FIG. 1.—The variation of the ACh concentration in μg./g. (black) and of the total amount (hatched) of ACh in μg./leaf in each of 10 consecutive leaves on a stem of *A. integra*. The values plotted are the means of 3 experiments. The relative age of the leaves is represented by the number under each block.

ACh Concentration in the Veins and the Blade.—For each experiment two leaves, a relatively young one adjacent to the terminal growing leaf and an older one situated at the lower end of the same branch, were used. The midrib and the secondary veins of each leaf were separated from the blade and then combined to form one sample called the "veins." The veins and the blade were cut into small pieces and the ACh was extracted and assayed. The results of 5 experiments are given in the upper section of Table I. In order to see if the distribution of acetylcholine among the veins and the blade of the leaves is affected by light, a series of similar experiments was carried out with leaves collected in darkness at a different season of the year, together with some leaves collected in bright daylight during the same season. The results obtained are given in the middle and lower sections of Table I. The veins had a higher ACh concentration than the blade in every leaf, and the ACh concentration in the veins and the blade of the old leaves were much lower than that in the young leaves. The ratio of ACh concentration in the veins to that in the blades of

TABLE I

ACETYLCHOLINE CONCENTRATION IN THE VEINS AND BLADES OF LEAVES OF *A. INTEGRA* (μg./g.)

Season and Period of Day of Collection	Young Leaves			Old Leaves		
	Veins	Blades	Veins/Blades	Veins	Blades	Veins/Blades
Leaves collected in daylight from Nov. 30, 1954, to Jan. 13, 1955	300	140	2·2	120	29	4·1
	450	150	3·0	57	20	2·9
	500	470	1·1	30	8	3·8
	500	340	1·5	25	7	3·6
	780	680	1·2	50	19	2·7
Average			1·8			3·4
Leaves collected in daylight from April 15 to 24, 1956	160	60	2·7	15	5	3·0
	220	120	1·8	23	7	3·3
	130	70	1·9	17	4	4·2
	210	100	2·1	14	5	2·8
Average			2·1			3·3
Leaves collected in darkness from April 5 to 24, 1956	220	55	4·0	16	3	5·3
	230	85	2·7	5	1	5·0
	150	75	2·0	7	2	3·5
	140	40	3·5	9	3	3·0
	360	220	1·6	9	2	4·5
Average			2·8			4·2

the young leaves was lower than that of the old leaves. Neither light nor the process of photosynthesis had any great effect on the distribution of ACh in the leaves.

Acetylcholine in the Stem

ACh Concentration of Various Segments of a Stem.—A young side branch with 10 to 12 leaves was taken, the leaves were removed, and the stem, which had a diameter of about 8 mm., was divided into 6 to 8 segments. Each segment, after its weight had been taken and its length measured, was extracted for ACh and assayed. The results of 3 such experiments are depicted in Fig. 2, which

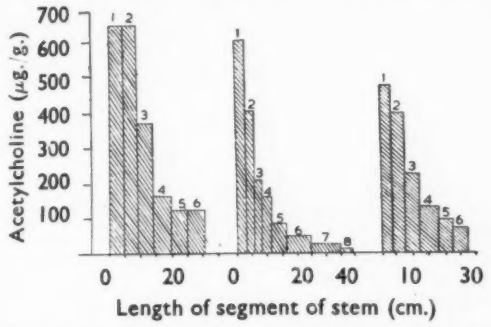

FIG. 2.—The ACh concentration of consecutive segments of three stems of *A. integra*. The relative age is represented by the numeral on each block ; the lowest numeral represents the youngest leaf. The width of each block is proportional to the length of each segment tested (cm.).

shows that the uppermost or youngest segment of the stem always had the highest ACh concentration and that the concentration in the other segments decreased with age.

Distribution of ACh in the Different Layers of Tissue of the Stem.—A transverse section of the young stem shows the cork, outer cortex, inner cortex, phloem, xylem and the pith which forms a conspicuous central cylinder. Microscopic examination showed that the pith cells contained an abundance of latex. In studying the distribution of ACh among these tissues, it was found practical to separate them into three layers: an outer or cortex-phloem layer which consisted of the outer and inner cortices without the cork but with part of the phloem which came off with the inner cortex, a middle layer which consisted mainly of the xylem, and the pith. A segment of young stem 5 to 8 mm. in diameter and about 30 mm. in length was used. It was first split into two halves from which the cortex-phloem layer was peeled off. The pith in the centre of each half was dissected out so as not to include the middle layer. In order to eliminate the possibility that some pith cells might still be attached to the inner side of the xylem and that part of the phloem tissue might have remained on the outer surface of the xylem, both the inner and the outer surfaces of the xylem layer were scraped until smooth. These three layers of tissue were then extracted separately for ACh. The results obtained from the examination of 8 different samples of Jack-fruit stem are given in Table II. The first 5 samples were taken from 5 different side branches

TABLE II

ACETYLCHOLINE CONCENTRATION OF DIFFERENT LAYERS OF TISSUE OF THE STEM OF *A. INTEGRA* (μg./g.)

Cortex-Phloem	Xylem	Pith	Pith/ Cortex-Phloem
25	8	64	2·6
85	120	600	7·1
53	16	130	2·4
65	24	330	5·1
160	70	320	2·9
130	36	410	3·2
50	12	360	7·2
35	8	130	3·3
Average 75	37	290	4·2

while the last 3 samples were from one branch. It shows that in every sample of the stem examined the pith has the highest and the xylem the lowest ACh concentration.

ACh Concentration of the Pith of Different Parts of Stem.—A young side branch of about 22 cm. in length was taken, and, after all the leaves on the stem had been removed, the stem was divided into 11 segments. Six alternate segments were used, while the others were discarded. The pith of each of these segments was dissected out and assayed for ACh. Table II gives the results, which

TABLE III

ACETYLCHOLINE CONCENTRATION IN THE PITH AND THE CORTEX-PHLOEM LAYER IN DIFFERENT PARTS OF THE STEM OF *A. INTEGRA*. THE VALUES (μg./g.) ARE THE MEANS OF THREE EXPERIMENTS AND WERE OBTAINED FROM DIFFERENT STEMS

Order of Segment by Age	Pith	Cortex-Phloem
1	130	190
2	110	130
3	78	70
4	75	42
5	57	41
6	45	32
7	—	31

show that the pith of the younger part of the stem has a higher ACh concentration than the older part.

ACh Concentration of the Cortex-Phloem Layer of Different Parts of Stem.—A young side branch of about 40 cm. in length and 8 mm.

in diameter was taken, and, after removing the leaves, the stem was divided into a number of segments. The two end segments and five other alternate segments in the intermediate region were used. The cortex-phloem layer of each segment was peeled off and then assayed for ACh. The results are given in Table III. The ACh concentration of the cortex-phloem layer of the stem also decreased with the age of the stem.

Acetylcholine in the Root

Presence of ACh in the Rootlets.—Nine different samples of rootlets varying from 1 to 2 mm. in diameter were taken from the side roots of a Jack-fruit tree on different dates. Each sample, after it had been cleaned with water and dried in air for a few minutes, was extracted for ACh. A mean value of ACh of 89 μg./g. was obtained (range, 65 to 120 μg./g.).

Distribution of ACh in the Tissues of the Root.—The external cork layer of the root dries up quickly and tends to break away from the outer cortex soon after collection. The cork and the outer cortex are thin, papery and crimson in colour and can be separated from the underlying tissues. There are a number of yellow spots or lenticel-like structures, each of about 1 to 3 mm. in diameter over the surface of the outer cortex as shown in Fig. 3. The number of yellow spots/unit area of the root surface varies from one part of the root to another, but as many as 7 spots have been counted within 1 sq. cm. on one root. They were seen to grow in the inner cortex layer. Such yellow spots are not normally seen in the roots of other plants. A transverse section of a spot showing a great number of loose "packing" cells alternating with bands of firm "closing" cells is shown in Fig. 4. These structures resemble

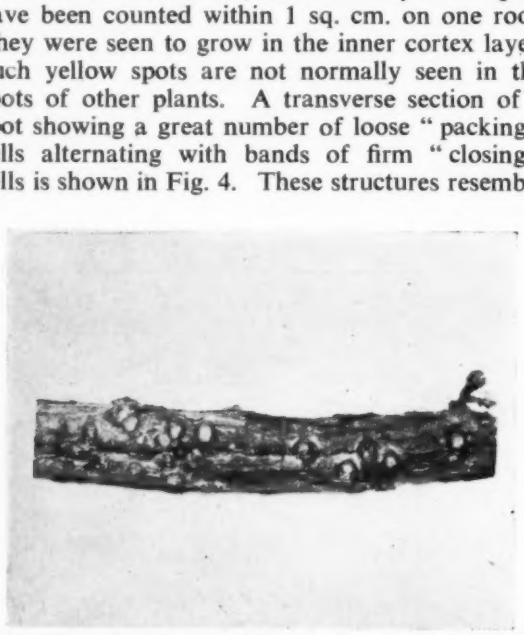

FIG. 3.—External appearance of the root of *A. integra* showing the yellow spots. About actual size.

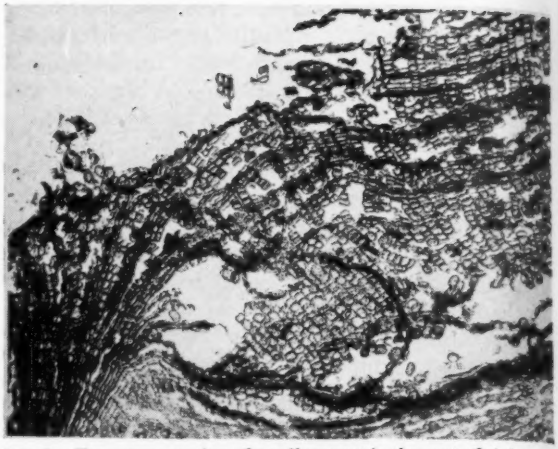

FIG. 4.—Transverse section of a yellow spot in the root of *A. integra*. Magnification, ×160.

those of the lenticels present in the stems of some plants. The phloem layer in the root is much thicker than it is in the stem and, hence, it can be separated from the inner cortex layer quite easily. The pith in the root is less extensive than in the stem.

The root-cork contained no ACh, or traces only. The other layers of the root tissue contained an appreciable amount of ACh, as given in Table IV.

TABLE IV

ACETYLCHOLINE CONCENTRATION IN DIFFERENT LAYERS OF TISSUE OF THE ROOT OF *A. INTEGRA* (μg./g.)

	Outer Cortex	Inner Cortex	Phloem	Xylem	Pith	Pith/Inner Cortex
	70	400	85	30	52	0·13
	120	250	45	36	38	0·15
	140	190	28	25	26	0·14
Average	110	280	53	31	39	0·14

The distribution of ACh in the root differed, however, from that in the stem. The ACh concentration in the pith of the stem (Table II) was 4.2 times greater than that in the cortex-phloem layer, whereas it was only one-seventh of that in the inner cortex layer of the root.

ACh in the Yellow Spots.—A short section of a side root about 10 mm. in diameter was taken and used immediately. The cork layer was removed and discarded and both the outer and inner cortex layers were separated and removed from the underlying tissues. Each layer was then divided into 2 portions, one containing all the yellow spots and the other containing the surrounding tissues. These four different portions of tissue were assayed for ACh. The results obtained are given

TABLE V

ACETYLCHOLINE CONCENTRATION IN DIFFERENT PARTS OF THE CORTEX OF THE ROOT OF *A. INTEGRA*. YS=THE YELLOW SPOTS (μg./g.)

	Outer Cortex			Inner Cortex		
	Without YS (I)	With YS (II)	II / I	Without YS (I)	With YS (II)	II / I
	60	220	3·7	50	460	9·2
	60	170	2·8	26	73	2·8
	15	35	2·3	20	110	5·5
	54	70	1·3	11	15	1·4
	60	240	4·0	70	600	8·6
Average	50	150	2·8	35	250	5·5

in Table V. It shows that, of both the outer and inner cortex layers of the root, the yellow spots have a much higher ACh concentration than the surrounding tissues.

DISCUSSION

The origin and function of substances in plants which have highly specific actions in animals are both a puzzle and a challenge. A study of the distribution of ACh in different tissues of *A. integra* throws some light on the site of formation of at least one pharmacologically important drug. The finding that young leaves and stems contain a much higher concentration of ACh than the corresponding older tissues suggests that ACh synthesis is confined to the period of growth.

The veins of the leaves have a higher concentration of ACh than the blade, and the ratio of the concentration in the veins to that in the blade is lower in the younger leaves. This might indicate that ACh is formed in the blades of the growing leaves and is then transported by the veins to the stem and roots.

In the stem, the pith has a higher concentration of ACh than the other layers of tissue ; it probably acts as a storage tissue. In the roots, on the other hand, the concentration of ACh is low in the pith and high in the inner cortex. The cortex contains some unusual yellow spots, in which ACh is concentrated. It is therefore possible that these yellow spots either synthesize ACh or absorb it from bacteria in the soil. On this hypothesis, ACh would be supplied by the roots to the actively growing leaves and stem by a diffusion process. In this case, however, it might be expected that the concentration gradient in the stem would be the opposite of that found, and the ACh concentration of the younger and more peripheral part of the stem would be lower than that of the older parts. This argument, together with the fact that there is no definite evidence for the synthesis of ACh by bacteria in the soil, or by the cells of the yellow spots, suggests that these spots are best regarded, like the pith in the stem, as storage structures. The growing leaves, then, are the most probable site of synthesis of ACh, but the question of how it is formed there still remains to be answered.

I should like to express my gratitude to Mr. J. Carrick, of the Department of Botany, University of Malaya, for his help in making the microscopic examination of the yellow spots on the root of *A. integra* and also for reading the manuscript. My thanks are also due to Professor E. M. Glaser and Mr. G. C. Whittow, of the Department of Physiology, and to Mr. A. S. C. Wan, of the Department of Pharmaceutics, University of Malaya, for reading the manuscript.

REFERENCES

Evans, W. C., and Partridge, M. W. (1953). *J. Pharm. Pharmacol.*, **5**, 293.
James, W. O. (1953). Ibid., **5**, 899.
James, G. M., and Thewlis, B. H. (1952). *New Phytol.*, **51**, 250.
Lin, R. C. Y. (1955). *Brit. J. Pharmacol.*, **10**, 247.

Brit. J. Pharmacol. (1957), **12**, 270.

THE EFFECT OF DINITROPHENOL, HYPOXAEMIA AND ISCHAEMIA ON THE PHOSPHORUS COMPOUNDS OF THE DOG HEART

BY

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The results reported in this paper indicate that dinitrophenol acts directly on the isolated heart, increasing its metabolic rate. It also produces heart failure associated with a low phosphocreatine content of the muscle but with no change in adenosine triphosphate, which may or may not be due to a relative hypoxia of the cardiac tissue. Experimental arterial hypoxaemia, if severe, produces a similar picture of heart failure with a decrease in phosphocreatine and no change in adenosine triphosphate. Ligation of the coronary arteries results in disappearance of the major part of the phosphocreatine within a few minutes regardless of whether or not ventricular fibrillation ensues; the adenosine triphosphate remains unchanged.

2:4-Dinitrophenol increases oxidative metabolism in animals several-fold by direct cellular action (Tainter and Cutting, 1933). It is also known to uncouple phosphorylation and oxidation in tissue homogenates or particulate systems derived therefrom (Loomis and Lipmann, 1948). Hence, if dinitrophenol were added to a well-oxygenated heart-lung preparation, one would expect to observe a failure of the heart. This failure would be due to lack of *production* of high-energy phosphate bonds, instead of lack of *utilization* of such bonds as is the case in heart failure due to barbiturates (Wollenberger, 1947; Fawaz and Hawa, 1953). This study was undertaken to determine to what extent observations made on homogenates and simpler systems can be applied to organs in activity. Since dinitrophenol may produce relative hypoxia by increasing metabolism, the effects of arterial hypoxaemia and ischaemia on the phosphorus compounds of the heart were also studied.

METHODS

Pentobarbitone anaesthesia was used in all animals. Heart-lung preparations were made by the conventional procedure except that the blood donors were anaesthetized with chloroform.

Detailed information for preparing samples from the left ventricle and for estimating the phosphorus compounds has been described previously (Fawaz and Hawa, 1953). In the present work, the labile nucleotide phosphorus was determined by hydrolysing the trichloracetic acid filtrate in N-HCl for 7 instead of 10 min. Experiments with pure crystalline adenosine triphosphate (ATP) showed that this was a sufficient period for 2/3 of the ATP phosphorus to be liberated. Our values are therefore slightly lower than before. In several control experiments and in a dinitrophenol experiment we compared the results obtained by this method with the paper chromatographic method for ATP determination. Nucleotides were precipitated from the trichloracetic acid filtrates with mercuric acetate (Kerr, 1940). After removal of the mercury by H_2S and evaporation to a small volume, the nucleotides were chromatographed on filter paper by the method of Magasanik, Vischer, Doniger, Elson, and Chargaff (1950). It was found that at least 90% of the 7 min. value, as determined on the trichloracetic acid filtrate, was accounted for by true ATP.

The phosphorus values in this paper are expressed not as mg./100 g. wet tissue but as % of the total acid-soluble phosphorus. Since it so happens that the total acid-soluble phosphorus is about 100 mg./100 g. the two methods of calculation give similar figures.

With the exception of Experiments 1 to 7 (Table I), the lungs in all dinitrophenol experiments were ventilated with pure oxygen. In addition, oxygen was bubbled through the venous reservoir by means of a Pyrex glass dispersion tube with fritted glass cylinder, foaming being prevented by the use of Dow Corning Antifoam A. The oxygen content of the arterial and coronary sinus blood was determined by the Van Slyke method, samples being taken at the beginning of the experiment and again just before the heart specimen was removed. Coronary sinus blood was

collected by means of a Morawitz cannula which also served to measure sinus flow. The cuff of the Morawitz cannula was inflated only when samples were withdrawn or measurements taken, because continued inflation produced widespread oedema and haemorrhagic necrosis of the left ventricle in some preparations. Hypoxaemia in the heart-lung preparation was produced by gradually diminishing the stroke-volume of the respiration pump. Ischaemia of the left apical ventricular area was produced in the intact animal by ligating the anterior descending and circumflex branches of the left coronary artery, leaving the septal branch intact. Ventricular fibrillation occurred a few min. after occlusion in 5 out of 7 experiments.

RESULTS

Dinitrophenol greatly increased the oxygen consumption of the heart. Although blood entering the heart from the venous reservoir was well oxygenated and the lungs were ventilated with oxygen, the systemic blood leaving the heart was dark and had a lower oxygen content (Table I). Measurements of coronary sinus flow and arteriovenous oxygen difference also showed increased oxygen utilization after dinitrophenol, but we consider this method of measuring oxygen consumption to be unreliable. The work of Katz, Jochim, and Weinstein (1938) and of Moe and Vischer (1940) shows that the coronary sinus flow does not represent a constant fraction of total coronary flow, nor is the oxygen content of the coronary sinus blood identical with that of the Thebesian blood. From our own observations it appears unlikely that the coronary sinus flow itself is accurately measured by the Morawitz cannula in the dog. When the heart was opened at the end of each experiment to make sure that the cannula was still in place, we discovered that in most dogs there were openings of varying magnitude in the walls of the sinus which would be blocked by inflating the rubber cuff of the cannula. The real purpose of the Morawitz cannula in our experiments was, therefore, to obtain samples of coronary sinus blood for oxygen determination; the estimates of coronary sinus flow and oxygen consumption have only qualitative significance.

Infusion of dinitrophenol sodium at the rate of 6 mg./min. usually produced no signs of failure during the first 10 to 15 min. Then there was a rise in venous pressure, usually not exceeding 2 cm. of water, with a decrease in systemic output but with no change of arterial blood pressure. Later, a point was reached when the blood pressure began to fall rapidly and cardiac arrest occurred in a few minutes. The decrease in systemic output does not necessarily reflect a fall in total left ventricular output because it might be compensated by a corresponding increase in coronary flow (Fawaz and Tutunji, 1957).

TABLE I

EFFECT OF DINITROPHENOL SODIUM ON THE ISOLATED DOG HEART

Phosphorus compounds are expressed as % of the total acid-soluble phosphorus.

Dinitrophenol-Sodium Infused (mg.)	Duration of Experiment (min.)	Systemic Output (ml./min.)		Coronary Sinus Flow (ml./min.)		O_2 Content of Arterial Blood (Vol. %)		O_2 Content of Coronary Sinus Blood (Vol. %)		Change in O_2 Consumption	Phospho-creatine-P	Inorganic-P	Labile Nucleotide-P
		Begin.	End	Begin.	End	Begin.	End	Begin.	End				
166	42	760	440								0	31·0	33·6
120	31	600	380								0	33·8	32·8
120	31	720	220								0	31·2	29·4
60	48	940	560								6·6	26·3	34·8
30	40	770	80								2·3	26·6	29·0
156	95	880	480								2·6	32·6	28·9
150	34	590	320								5·5	28·4	33·5
150	44	840	190								0	29·2	35·2
150	31	770	300								3·7	28·6	32·6
100	133	790	250								8·9	24·3	31·2
150	31	680	500	39	220	16·7	9·4	6·0	4·3	+170%	0	29·0	32·2
150	29	740	300	31	65	16·7	12·0	9·4	4·3	+120%	6·0	28·6	33·6
150	41	550	290	46	110	19·7	13·7	6·9	5·1	+163%	5·8	30·6	26·6
120	37	600	140	17	—	17·6	8·6	6·0	2·6		2·2	35·6	27·0
150	40	880	0	46	190	17·7	12·1	9·9	8·6	+85%	0	27·7	35·7
150	30	760	290	20	47	16·5	11·8	5·7	5·2	+44%	0	35·3	27·9
150	29	640	500	24	200	15·9	8·9	4·4	1·9	+407%	0	39·7	26·5
150	34	740	360	13	54	27·4	22·9	8·0	4·0	+305%	4·3	33·3	26·4
150	34	800	420	18	49	26·5	20·3	6·2	3·5	+126%	0	33·7	26·8
120	36	670	380	47	173	16·7	8·8	5·3	4·4	+42%	4·8	34·1	17·1
150	36	990	120	30	—	25·1	16·7	6·2	7·0		5·4	30·8	28·2
150	32	860	580	34	—	20·5	15·1	8·0	2·7		0	36·0	28·4
120	33	800	540	24	113	21·8	16·0	6·2	5·4	+220%	0	32·2	25·3
										Average ± S.E.	2·5 ± 0·6	31·2 ± 0·8	29·7 ± 0·9

TABLE II

EFFECT OF HYPOXAEMIA ON THE ISOLATED DOG HEART

Phosphorus compounds are expressed as % of the total acid-soluble phosphorus.

Duration of Hypoxaemia (min.)	Systemic Output (ml./min.)		Coronary Sinus Flow (ml./min.)		O_2 Content of Arterial Blood (Vol.%)		O_2 Content Coronary Sinus Blood (Vol.%)		Change in O_2 Consumption	Phospho-creatine-P	Inorganic-P	Labile Nucleotide-P
	Begin.	End	Begin.	End	Begin.	End	Begin.	End				
54	650	320	28	108	17·5	1·8	6·8	0·6	−57%	0	35·7	30·2
70	900	530	30	48	21·0	10·2	4·0	4·2	−44%	20·7	17·6	36·4
55	820	440	53	105	13·4	7·6	4·9	2·3	+24%	15·5	24·6	27·0
51	800	690	26	38	20·0	14·7	4·0	4·9	−11%	21·4	13·3	32·6
62	560	390	24	36	14·7	9·3	4·9	4·9	−32%	22·5	15·5	33·2
50	640	380	173	149	12·1	2·2	5·0	1·1	−87%	6·3	28·1	26·7
40	600	300	48	216	10·5	1·8	3·0	0·7	−34%	5·6	31·3	28·0
45	750	630	26	50	12·9	7·3	3·8	3·4	−18%	21·7	18·5	28·2
45	612	570	68	108			5·0	3·4		12·0	24·4	31·0
22	468	468	36	62			9·3	4·0		17·8	18·9	31·0

Gradual arterial hypoxaemia produced a similar sudden terminal fall in blood pressure. In the dinitrophenol experiments, the heart specimen was removed just after the sudden onset of failure, and in the hypoxaemia experiments when the coronary sinus blood was judged to be as dark as in the dinitrophenol experiments (Table II).

Dinitrophenol failure is associated with a marked diminution in the phosphocreatine content of heart muscle. The same thing is observed in marked hypoxaemia. The decrease in phospho-creatine in the dinitrophenol-treated heart might therefore be due to relative hypoxia secondary to the increase in metabolism. However, it can be seen from a comparison of Tables I and II that the oxygen content of the coronary sinus blood in the hypoxaemia experiments fell to extremely low levels before the creatine-phosphate reached the low values observed in dinitrophenol experiments with higher oxygen concentrations in the coronary sinus blood. There is an element of uncertainty regarding the adequacy of the control hypoxaemia experiments because the criteria for hypoxia in tissues respiring normally may not be the same as those in tissues with an abnormally high oxidative rate.

Ischaemia caused by ligating the coronary vessels resulted in a disappearance of most of the phosphocreatine within a few minutes, whether or not ventricular fibrillation occurred. This is shown in Table III. Here again the labile ATP phosphorus remained essentially unchanged.

A control series of 9 normal dog hearts (not heart-lung preparations) were analysed for phosphorus compounds during the same period of time in which the above results were obtained. The figures were: phosphocreatine-P, 15.9 ± 0.5; inorganic-P, 19.3 ± 1.4; labile "nucleotide"-P, 31.4 ± 0.9.

TABLE III

EFFECT OF ISCHAEMIA ON THE PHOSPHORUS COMPOUNDS OF DOG HEART IN INTACT OPEN-CHEST ANIMALS

Phosphorus compounds are expressed as % of the total acid-soluble phosphorus.

Duration of Ischaemia (min.)	Interval between Ligation and Vent. Fibrillation (min.)	Phospho-creatine-P	Inorganic-P	Labile Nucleotide-P
2	1	2·0	36·8	26·8
4	—	2·8	33·4	27·8
4	—	2·0	32·6	30·2
2·2	1·8	1·6	32·7	28·7
1·5	1·5	2·3	28·4	35·1
2	1	3·3	35·2	22·5
4	3	3·0	37·8	32·2
	Average ± S.E.	2·4 ± 0·2	33·8 ± 1·2	29·0 ± 1·5

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REFERENCES

Fawaz, G., and Hawa, E. S. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 277.

—— and Tutunji, B. (1957). *Brit. J. Pharmacol.*, **12**, 273.

Katz, L. N., Jochim, K., and Weinstein, W. (1938). *Amer. J. Physiol.*, **122**, 252.

Kerr, S. E. (1940). *J. biol. Chem.*, **132**, 147.

Loomis, W. F., and Lipmann, F. (1948). Ibid., **173**, 807.

Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E. (1950). Ibid., **186**, 37.

Moe, G. K., and Vischer, M. B. (1940). *Blood, Heart and Circulation* (*Publ. Amer. Ass. Advanc. Sci.*), 100.

Tainter, M. L., and Cutting, W. C. (1933). *J. Pharmacol.*, **48**, 410.

Wollenberger, A. (1947). *Amer. J. Physiol.*, **150**, 733.

Brit. J. Pharmacol. (1957), **12**, 273.

THE MECHANISM OF DINITROPHENOL HEART FAILURE

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Hypoxaemia, resulting from increased tissue metabolism, is an important factor in dinitrophenol failure in the conventional heart-lung preparation. Improved oxygenation of the blood by a technique described in this paper prolongs the life of dinitrophenol-treated hearts. Dinitrophenol acts very rapidly; oxygen consumption and coronary flow increase in a few minutes and the increase is proportional to the dose. The increase in oxygen consumption diminishes with time. Dinitrophenol decreases the phosphocreatine content of the heart, even when there is no failure or hypoxia. There is no evidence that dinitrophenol failure can be due to a decrease of phosphocreatine or adenosine triphosphate content of the heart, although this is to be expected in view of the observed " uncoupling " action of dinitrophenol.

Results reported by Fawaz, Hawa, and Tutunji (1957) do not answer the following questions: (*a*) Are the low phosphocreatine values associated with dinitrophenol heart failure due to the direct " uncoupling " action of dinitrophenol or are they caused by hypoxaemia resulting from increased metabolism? (*b*) Is the decrease in phosphocreatine the cause of the failure? Furthermore, serious objections were raised by us against the measurement of coronary sinus flow by means of the Morawitz cannula technique. The importance of measuring coronary flow accurately cannot be over-emphasized, because a decrease in systemic output may simulate failure, when in reality a corresponding increase in coronary flow may be taking place.

METHODS

Coronary flow was measured by the Rodbard technique (1953) as adapted to the heart–lung preparation (Badeer, 1955). We used this standard procedure in one set of experiments, ventilating the lungs with pure oxygen and bubbling oxygen through the venous reservoir. In another set of experiments the oxygenation of the blood was improved by diverting the coronary venous blood to the venous reservoir for oxygenation. This modification is illustrated in Fig. 1. The heart–lung preparation was allowed to run for a short time with the venous inflow cannula inserted into the distal end of the left pulmonary artery. The venous inflow tube was then clamped and the peripheral resistance reduced. The proximal end of the right pulmonary artery was cannulated and the coronary venous blood, collected in a beaker, was returned to the venous reservoir. The distal end of the right pulmonary artery was connected to the venous reservoir. The venous inflow was then re-established and the peripheral resistance adjusted so that the blood pressure was maintained at 10 cm. Hg. The venous inflow level was kept at 30 to 35 cm. above the left auricle and this yielded a systemic output of 600 to 900 ml./min., an amount similar to that obtained with the conventional heart-lung prepara-

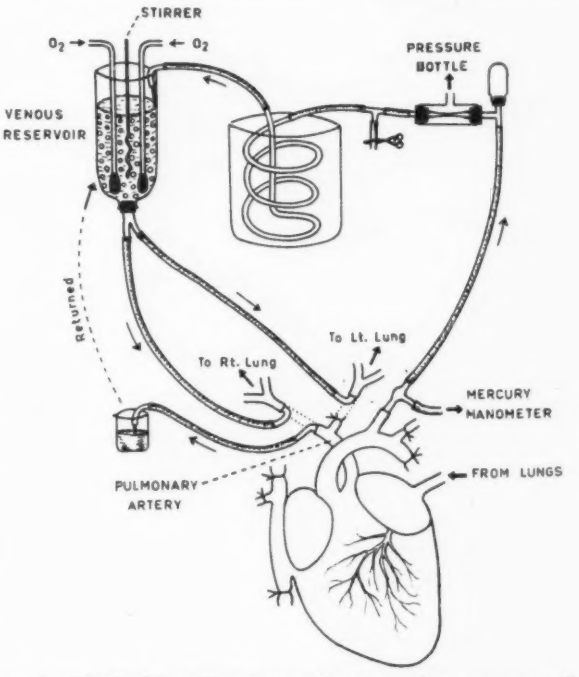

FIG. 1.—Modified heart-lung preparation to permit measurement of total coronary flow and myocardial oxygen consumption, and to improve arterial oxygen saturation in dinitrophenol experiments.

tion. The right heart was made to pump against a pressure of 16 cm. of water. This level was chosen because higher pressures resulted in failure of the right heart. Good arterial saturation was ensured by bubbling oxygen by means of two Pyrex glass dispersion tubes with fritted cylinders inserted at the bottom of a column of blood 7 cm. in diameter and 14 cm. in depth in the venous reservoir.

Dinitrophenol sodium was added slowly to the reservoir at 3 min. intervals in doses of 45 mg. If more than a total of 90 mg. was used, an interval of 10 min. was allowed to elapse before the third dose was given.

The phosphorus compounds were determined by the method of Fawaz *et al.* (1957). Adenosine triphosphate (ATP) was estimated chromatographically on several dinitrophenol-treated hearts and was found to be about 90% of the 7 min. hydrolysis value as reported in the tables.

Results

Experiments Utilizing the Rodbard Technique

Experiments Terminated Before Failure.—In this group, with a dose of 60 to 90 mg. of dinitrophenol, the experiment was terminated before any evidence of gradual or sudden failure developed. There was no decrease in total left ventricular output or of work performed after 40 min. There was an increase in coronary flow which was greater than that observed in control experiments of the same duration. The average oxygen content of coronary arterial blood at the end of the experiment was 13.1 vol.% as compared to 18.4 before dinitrophenol was added. The oxygen content of coronary venous blood did not change. The phosphocreatine values were about half the values obtained in similar control experiments and the labile nucleotide phosphorus did not change (Table I).

Experiments Terminated at the Onset of Sudden Failure.—The preparations survived from 10 to 41 min. after a dose of 90 mg. This confirms the observation of Gruhzit and Farah (1955) that there is great variation in the susceptibility of different hearts to dinitrophenol. There was again no decrease in output or performance of the heart before the onset of sudden failure. However, the average arterial oxygen saturation (8.5 vol.%) and the coronary venous oxygen saturation (3.1 vol.%) at the end of the experiment were significantly lower than the figures obtained in experiments terminated before failure. The phosphocreatine content was also lower, averaging about one-fourth the normal figures. The ATP values were insignificantly lower. Table I summarizes these results, and it seemed that, in this group of experi-

TABLE I

EFFECT OF DINITROPHENOL SODIUM ON THE DOG HEART-LUNG PREPARATION PERFORMED WITH THE RODBARD TECHNIQUE

Total Dose of Dinitrophenol Na	Duration of Expt. in Min.	Coronary Flow (ml./min.)		Total Left Vent. Output (ml./min.)		Change in Work Performed (%)	Change in O_2-Consumption (%)	O_2-content of Arterial Blood (Vol.%)		O_2-content of Coronary Venous Blood (Vol.%)		Phosphorus Compounds of Heart Muscle (% of Total Acid-soluble-P)		
		Begin.	End	Begin.	End			Begin.	End	Begin.	End	Phosphocreatine	Inorganic Phosphorus	Labile Nucleotide-P (2/3 of ATP)
Experiments terminated before sudden failure														
60	40	60	130	774	947	+23	+97	15·3	12·6	5·4	3·6	16·4	22·3	29·6
60	40	64	268	694	931	+39	+86	17·4	10·8	4·8	5·2	20·0	22·4	27·4
60	40	48	104	521	954	+98	+58	19·0	14·5	8·3	6·7	10·1	19·8	33·4
90	40	52	220	621	554	−14	+103	17·9	11·9	6·3	6·3	8·4	24·0	32·4
90	40	80	252	700	1,381	+117	+129	17·9	15·2	4·7	5·6	12·1	19·7	32·5
90	40	128	260	837	1,126	+37	+57	18·6	15·1	5·0	4·6	16·5	17·4	31·3
90	40	46	300	411	774	+93	+161	19·8	10·9	6·1	5·5	11·3	20·2	34·7
90	40	40	320	452	700	+52	+242	17·9	10·7	5·4	5·4	9·7	19·0	36·7
90	40	68	240	819	972	+20	+35	22·2	16·2	6·2	9·3	9·8	23·2	32·5
Average ±S.E.		65	233	659	926			18·4	13·1	5·8	5·8	12·7±0·9	20·9±0·7	32·3±0·9
Experiments terminated at onset of sudden failure														
90	28	138	308	1,110	1,131	+5	−69	18·8	4·5	7·2	2·9	1·0	31·2	30·4
90	20	96	288	826	917	+5	+50	21·6	9·3	5·6	1·4	1·4	30·8	29·3
90	15	132	292	505	441	−32	−47	20·5	6·2	5·6	2·7	5·4	35·8	25·2
90	24	120	480	456	840	+85	+121	16·4	6·4	8·0	1·8	8·9	24·2	32·0
90	15	84	464	668	1,230	+97	+218	16·4	8·1	9·0	3·8	12·5	25·2	28·8
90	10	92	420	605	860	+42	+268	19·7	12·5	9·9	4·5	15·9	25·6	29·0
90	41	68	224	315	350	+3	+136	20·0	11·4	5·3	0·9	2·0	32·1	27·2
90	38	76	440	638	1,384	+128	+88	17·9	12·3	7·1	8·8	10·3	19·6	30·8
90	33	76	412	636	1,029	+80	+164	18·1	6·2	9·9	2·0	0·8	34·7	23·3
90	11	48	300	605	750	+24	+263	17·6	8·2	6·6	1·8	1·2	38·0	28·2
Average ±S.E.		93	363	636	893			18·7	8·5	7·4	3·1	5·9±1·8	29·7±1·5	28·4±0·8

TABLE II

EFFECT OF DINITROPHENOL SODIUM ON THE DOG HEART-LUNG PREPARATION PERFORMED WITH THE MODIFIED TECHNIQUE

Total Dose of Dinitrophenol Na	Duration of Expt. (min.)	Coronary Flow (ml./min.)			Total Left Vent. Output (ml./min.)			Change in Work Performed (%)		Change in O_2-Consumption (%)		O_2-content of Arterial Blood (Vol.%)			O_2-content of Coronary Venous Blood (Vol.%)			Phosphorus Compounds of Heart Muscle (% of Total Acid-soluble-P)		
		Begin.	Middle	End	Begin.	Middle	End	Middle	End	Middle	End	Begin.	Middle	End	Begin.	Middle	End	Phospho-creatine	Inorganic-P	Labile Nucleotide-P (2/3 of ATP)
Short-term experiments terminated before sudden failure																				
90	23	23		192	709		727		−20		+373	18·7		16·8	6·2		9·8	16·4	19·5	33·3
90	28	52		252	681		421		−31		+419	15·6		14·9	4·6		5·2	20·0	19·7	31·4
135	30	58		264	520		494		+5		+206	17·8		16·1	7·0		8·5	11·5	24·1	31·6
135	29	41		366	705		631		−20		+298	14·5		9·0	4·4		4·5	5·5	25·8	33·6
135	31	38		276	678		556		−33		+198	21·7		16·8	6·6		10·6	10·2	19·7	34·6
135	24	72		440	739		724		−13		+240	16·0		14·8	8·8		10·7	10·3	25·2	30·4
135	26	50		424	715		826		+13		+439	22·2		18·7	12·6		12·6	12·2	23·0	30·0
Average ±S.E.		48		316	678		626					18·1		15·3	7·2		8·8	12·3±1·8	22·4±1·0	32·1±2·1
Long-term experiments terminated before sudden failure																				
90	87	52	280	300	657	764	534	+16	−23	+253	+221	19·0	15·4	13·6	5·9	6·8	6·3	16·2	20·5	31·2
90	159	40	110	116	788	601	320	−27	−65	+143	+56	19·7	17·9	17·2	7·6	7·2	10·7	20·5	18·2	36·2
90	152	52	286	252	549	495	493	−13	−13	+228	+66	17·1	11·1	9·9	8·9	6·2	7·1	9·9	24·4	30·4
90	120	94	224	196	1,267	966	636	−28	−54	+108	+77	13·1	12·6	12·6	5·9	6·3	6·5	13·0	18·8	34·6
180	94	30	390	300	717	621	438	−20	−44	+540	+239	18·6	9·4	13·1	7·4	4·2	9·3	14·7	22·4	27·6
180	116	30	296	276	777	734	472	−5	−45	+451	+266	20·4	18·7	20·4	6·8	11·1	15·0	7·9	22·5	32·2
180	83	44	300	200	996	533	328	−53	−60	+437	+169	18·3	15·3	16·1	3·6	3·7	7·4	12·6	22·2	30·8
Average ±S.E.		49	269	234	822	693	460					18·0	14·3	14·7	6·6	6·5	8·9	13·5±1·6	21·3±0·8	31·9±1·1
Experiments terminated at onset of sudden failure																				
90	96	68	232	240	675	831	530	+27	−29	+113	−3	16·6	13·6	10·2	5·7	6·8	7·2	11·4	23·2	33·4
90	134	38		168	570		257		−59		−19	20·0		18·4	5·7		15·8	10·8	20·0	34·7
90	152	32	194	116	539	381	200	−34	−70	+253	+20	19·1	11·9	19·1	7·6	5·2	15·3	13·8	23·1	31·7
90	115	56	166	200	1,298	1,143	292	−17	−85	+67	−10	22·4	20·6	20·6	7·3	12·1	16·8	4·1	37·0	23·6
135	106	42	312	216	646	655	478	0	−32	+232	+113	20·2	13·5	9·0	7·2	7·7	3·6	4·7	21·6	34·8
180	117	40	380	120	668	586	192	−18	−75	+415	+48	22·8	15·2	20·1	9·6	8·0	13·6	13·9	19·2	31·5
180	92	44	340	192	1,368	890	319	−44	−82	+331	+156	18·0	13·7	16·6	4·7	6·3	8·8	3·3	26·2	30·6
Average ±S.E.		46	271	179	823	748	324					19·9	14·8	16·3	6·8	7·7	11·4	8·9±1·8	24·3±2·3	31·4±1·4

TABLE III

CONTROL EXPERIMENTS

The first three experiments were performed by the Rodbard technique, the rest by the modified technique. * = Spontaneous failure. † = Barbiturate failure.

Duration of Expt. (min.)	Coronary Flow (ml. min.)			Total Left Vent. Output (ml./min.)			Change in Work Performed (%)		Change in O_2-consumption (%)		O_2-content of Arterial Blood (Vol.%)			O_2-content of Coronary Venous Blood (Vol.%)			Phosphorus Compounds of Heart Muscle (% of Total Acid-soluble-P)		
	Begin.	After 60 min.	End	Begin.	After 60 min.	End	After 60 min.	End	After 60 min.	End	Begin.	After 60 min.	End	Begin.	After 60 min.	End	Phospho-creatine	Inorganic-P	Labile Nucleotide-P (2/3 of ATP)
40	132		132	841		829		+1		0	19·5		19·5	5·1		5·1	22·5	17·4	32·0
40	92		196	941		1,080		+11		−4	17·7		16·9	8·2		12·6	23·1	15·7	31·2
40	140		152	634		733		+18		+43	15·7		17·5	8·7		8·3	26·8	15·5	26·1
122	24	50	66	266	499	409	+100	+62	+28	+11	18·2	18·1	18·2	6·8	11·1	13·6	18·8	19·9	27·8
122	36	74	202	784	801	762	+3	−2	−8	+1	22·6	23·0	21·8	8·3	16·6	19·3	27·7	12·0	26·4
120	42	63	208	886	1,173	969	+37	+9	+5	+65	20·8	20·0	21·1	6·5	10·0	16·3	24·4	15·3	30·4
121	40	56	72	908	1,226	1,243	+45	+38	+38	+24	18·0	18·0	20·4	5·4	5·6	11·7	22·5	18·5	32·8
44	30		26	261		77		−78		−43	21·6		20·8	5·4		9·9	29·4	11·8	30·1*
120	30		142	849		288		−95		−6	21·7		17·4	8·1		14·7	20·4	13·8	33·6†
110	42		114	562		211		−66		−13	19·0		17·0	9·9		14·1	22·9	12·4	32·0†
Average ±S.E.	61	61	131	693	925	660					19·5	19·8	19·1	7·2	10·8	12·6	23·9±1·0	15·2±0·9	30·2±0·8

ments, the heart failure was attributable to arterial hypoxaemia, probably due to failure of the lung to do its share in the oxygenation of coronary blood.

Experiments with the Modified Rodbard Technique

Short-term Experiments Terminated Before Failure.—The dose of dinitrophenol was increased to 90 to 135 mg. There was little change in total left ventricular output or work performed due to the drug, but there was a significant increase in oxygen consumption (200 to 400%). The arterial oxygen saturation at the end of the experiment was satisfactory and the oxygen saturation of coronary venous blood was high. The phosphocreatine values were about 50% of normal and there was no change in ATP (Table II).

Long-term Experiments Terminated Before Failure.—These experiments were allowed to run for a period of 83 to 160 min. with doses of 90 to 180 mg. of dinitrophenol. The increase in oxygen consumption after 30 to 60 min. was greater than at the end. There was also a decrease in total left ventricular output towards the end of the experiment, part of which may have been due to spontaneous failure. Here again arterial and venous oxygenation were satisfactory. Phosphocreatine values were about 50% of normal and there was no change in ATP (Table II).

Experiments Terminated at the Onset of Failure.—The dose of dinitrophenol was 90 to 180 mg. and the survival period was 92 to 152 min. These results, given in Table II, demonstrate again the effect of proper oxygenation in prolonging the survival period of heart-lung preparations treated with dinitrophenol. The oxygen consumption at the middle of the experiment was greater than at the end, and there was a decrease in total output and work performed towards the end. Arterial and venous oxygen saturations were very satisfactory. The phosphocreatine values were slightly less than half the normal.

Control Experiments

These were carried out on heart-lung preparations without dinitrophenol, and were allowed to run for 40 to 120 min. Three experiments were performed with the Rodbard technique, the rest with the modified procedure (Table III). One heart failed spontaneously, two were made to fail by the addition of pentobarbitone. There was a general increase in coronary flow and a corresponding increase in the oxygen saturation of coronary venous blood (average 12.6 vol.%). The phosphocreatine values were consistently high, averaging

24% of the total acid-soluble phosphorus. The changes in oxygen consumption and work performed as the experiment proceeded were not significant.

Discussion

One important fact which emerged from the foregoing experiments is that hypoxaemia, due to increased tissue metabolism, is an important factor in dinitrophenol failure in the conventional heart-lung preparation. Improved oxygenation of the blood resulted in prolongation of the life of the preparation. To what extent hypoxaemia has played a role in results of experiments published by others is difficult to say, but neither Rothlin, Taeschler and Cerletti (1955) nor Gruhzit and Farah (1955) mentioned observing hypoxaemia. Yet it is common knowledge that the oxygen saturation of the blood in an ordinary heart-lung preparation without dinitrophenol often markedly improves when the lungs are ventilated with oxygen instead of air.

It is surprising that the lungs in a heart-lung preparation are not able to oxygenate blood serving only these two organs, when *in situ* they serve the needs of the whole body. Could this "unphysiological" behaviour of the isolated lungs be due to the fact that the lungs *in situ* receive their nutrition through the bronchial arteries ?

Dinitrophenol acts very rapidly in increasing oxygen consumption and coronary flow. These effects are visible a few minutes after a dose of 45 mg. of the drug is added. The increase in the coronary flow and the corresponding decrease in systemic output may be so great as to simulate heart failure if no accurate method is available for measuring coronary flow. This is particularly true of short-term experiments. In general, the increase in oxygen consumption is proportional to the dose of dinitrophenol ; in long-term experiments the effect fades away towards the end, even if a correction is made for the decrease in work performed. We have no explanation for this finding.

Dinitrophenol decreases the phosphocreatine content of cardiac muscle in the heart-lung preparation. There is a 50% decrease even when the factor of hypoxaemia is eliminated and no signs of failure are present. Yet the conclusion cannot be drawn that dinitrophenol failure is due to its action of uncoupling phosphorylation and oxidation with the resulting unavailability of high-energy phosphate bonds. In the short-term experiments with proper oxygenation, terminated before failure, dinitrophenol exerted its maximum activity, increasing metabolism 2 to 4 times. This is clear evidence that the drug had diffused into the cells. The doses given, although in excess of those used in homogenates and isolated enzyme systems to uncouple phosphorylation and oxidation, did not produce failure at the height of their metabolic action. Furthermore, in the long-term experiments with bigger doses and proper oxygenation, terminated before sudden failure, where there was a gradual decrease in output and work performed towards the end, the phosphocreatine values were not lower than in the previous short-term experiments. Even in the experiments with proper oxygenation terminated during heart failure, the phosphocreatine values were not significantly lower than in the experiments terminated before sudden failure. It is therefore not possible to correlate dinitrophenol failure with a decrease in phosphocreatine content. What has been said of phosphocreatine applies also to adenosine triphosphate, which does not change after giving dinitrophenol. The results of these experiments are similar to those obtained with fluoroacetate on the heart-lung preparation (Fawaz, 1956). Fluoroacetate is also a substance which blocks the Krebs cycle at the citrate stage and would be expected to produce failure due to lack of availability of high-energy phosphate bonds. We found that fluoroacetate produced failure to a degree which was proportional to the dose of the drug. Citrate accumulation in the heart was also proportional to the dose of fluoroacetate ; phosphocreatine decreased, but there was little correlation between the degree of failure and the decrease in phosphocreatine. Here again, ATP did not change during failure.

To summarize, heart failure produced by hypoxaemia, ischaemia due to ligation of the coronary arteries, dinitrophenol with or without tissue anoxia, or by fluoroacetate is associated with a significant decrease in phosphocreatine but no change in the ATP content of the heart. Phosphocreatine breakdown may or may not have the function of replenishing the ATP reserves, but it is difficult to visualize why the heart should not continue its action if, as in all these cited cases of failure, its ATP stores are still intact. It is thus difficult to reconcile these findings with the accepted view that ATP plays the dominant role in supplying energy for cardiac muscle contraction, and one must be careful not to assume that results obtained *in vitro* with simplified systems should necessarily apply *in vivo*.

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REFERENCES

Badeer, H. (1955). *Amer. J. Physiol.*, **183**, 119.

Fawaz, G. (1956). *Arch. exp. Path. Pharmak.*, **228**, 377.

—— Hawa, E. S., and Tutunji, B. (1957). *Brit. J. Pharmacol.*, **12**, 270.

Gruhzit, C. C., and Farah, A. E. (1955). *J. Pharmacol.*, **114**, 334.

Rodbard, S., Graham, G. R., and Williams, F. (1953). *J. appl. Physiol.*, **6**, 311.

Rothlin, E., Taeschler, M., and Cerletti, A. (1955). *Circulation Res.*, **3**, 32.

Brit. J. Pharmacol. (1957), **12**, 279.

THE EFFECT OF DIURETICS ON THE WATER EXCRETION OF PROTEIN DEFICIENT RATS

BY

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Adult rats kept for eleven weeks on a diet deficient in protein lost weight and some developed scrotal oedema. The retention of bromsulphthalein was increased, but the thymol turbidity test was unaffected; the apparent plasma volume was increased.

Water diuresis in the protein deficient animals was impaired. There was no apparent delay in the mean rate of water absorption from the whole gastro-intestinal tract although a delayed absorption of water from the intestine was found in some animals. The concentrations of total plasma proteins and plasma albumin were low as compared with normal animals, but the plasma sodium levels were within normal limits. The inulin clearance (glomerular filtration rate) of the animals on the protein-deficient diet was significantly lower than that of the controls.

In normal rats, aminophylline and acetazolamide were diuretic. Caffeine and sodium benzoate did not increase the urine output and mersalyl was antidiuretic. In the protein deficient rats, cortisone acetate increased the water diuresis. Caffeine and sodium benzoate, aminophylline and acetazolamide did not significantly increase this response, mersalyl had an antidiuretic effect. Cortisone acetate increased the food and water intake of the protein deficient rats; it also increased the glomerular filtration rate.

It is now well established that animals kept on low protein diets have an impaired water diuresis (Dicker, Heller, and Hewer, 1946; Heller and Dicker, 1947; Leslie and Ralli, 1947; Guggenheim and Hegsted, 1953; Schnieden and Blackmore, 1955a). Rats kept on a protein deficient diet also have an increased total body water and are oedematous (Haigh and Schnieden, 1956). We have investigated the effects of diuretics on such animals to see whether water diuresis could be restored. In addition to the conventional drugs (mercurial and xanthine diuretics), cortisone and corticotrophin were also tested because these hormones have a diuretic effect in nephrosis (Luetscher, Deming, Harvey, Lew, and Poo, 1950; Luetscher, Deming and Johnston, 1951). Nephrosis, like nutritional protein deficiency, is characterized by a low plasma protein level and oedema.

METHODS

Animals.—Male albino rats weighing 180 to 240 g. were used. Diets and water were unrestricted.

Control Diet.—This consisted of: wheat seed 22.7, barley meal 19.5, linseed cake (foreign) 19.5, decorticated ground nut meal 19.5, dried separated milk 2.4, white fish meal 4.8, meadow hay 9.8, cod-liver oil 1.8. This diet contained 25.6% protein (crude), 3.9% fat (ether extract), and 45.2% carbohydrate (Woodman, 1952). It provided approximately 330 cal./100 g. and 7.8 g. protein (crude)/100 cal.

Protein Deficient Diet.—This consisted of: casein 0.5, maize starch 80, hardened ground nut oil 12, Steenbock's salt mixture (Hawk and Bergeim, 1942) 4.5, cod liver oil 3.0, supplemented by 50 mg. vitamin B complex powder (Crookes Laboratories/100 g. diet. It contained 0.4% thiamine, 0.5% riboflavine, 0.18% pyridoxine, 4% nicotinamide, and 0.0014% pantothenic acid. 3 mg. tocopherol acetate/100 g. diet was also added. This diet provided approximately 300 cal./100 g. and 0.2 protein/100 cal.

Measurement of Food Intake.—The daily intake of food was estimated by placing groups of 6 rats in suspended cages. Weighed amounts of food in excess of the daily requirement were provided. Some scattering of food could not be prevented, so the cages were suspended over a tray lined with filter paper and the scattered food separated from the faeces each morning and added to food not consumed.

Measurement of Water Intake.—Rats were placed in individual metabolism cages fitted with graduated drinking tubes. The amount of water drunk by each animal in 24 hr. was noted.

Plasma Proteins.—A biuret method was employed for estimation of total plasma protein and plasma albumin (Hiller, 1927; Peters and Van Slyke, 1932).

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Plasma Sodium.—Heparinized plasma was obtained from blood taken from a carotid artery. A flame photometer was used for the estimation of sodium.

Plasma Volume (Evans Blue Space).—This was estimated according to the procedure used by Ginsburg (1954). The rats were allowed food and water up to the beginning of the experiment. They were then anaesthetized with ether and cannulae were inserted into a jugular vein and a carotid artery. Evans blue was injected into the jugular vein and blood samples were obtained from the carotid artery.

Haematocrit Estimations.—Wintrobe's technique and tubes were used.

Liver Function Tests.—Thymol turbidity tests were done according to McLagan (1944). Bromsulphthalein retention in rats was estimated by a modification of the method of Ahmed and Frazer (1952). A polythene cannula was inserted into the external jugular vein of the rat under ether anaesthesia and brought out through the skin between the ears (see Ginsburg and Heller, 1953). The animal was later allowed access to food and water until the start of the test 24 hr. later. Bromsulphthalein (25 mg./kg. body weight) was injected intravenously and 25 min. later the animal was quickly anaesthetized with ether. A siliconed polythene cannula was inserted into the carotid artery. Blood was collected from the carotid artery into heparinized tubes exactly 30 min. after the bromsulphthalein injection. The technique used makes it possible to obtain plasma completely free from haemolysed cells. The dye concentration in the plasma was estimated electrophotometrically.

Response to Water Administration (Water Diuresis).—Rats were randomly divided into groups before being put on diet and kept under identical environmental conditions before and during the experiments. The procedure of Schnieden and Blackmore (1956) was used.

Gastro-intestinal Absorption.—The procedure described by Schnieden and Blackmore (1955b), which is based on the method of Heller and Smirk (1932), was used. The animals were killed 45 min. after administration of their second dose of water.

Insulin Clearance (Glomerular Filtration Rate).—The method of Dicker and Heller (1945) was used, but more water was given so that the estimation was carried out when the animal had a water load of approximately 7% of its body weight. Inulin was estimated by the resorcinol method of Schreiner (1950).

Materials.—Corticotrophin (Crookes), cortisone acetate (Merck) and cortisone suspending fluid were supplied by the Medical Research Council. Other preparations used were mersalyl (Bayer), aminophylline (Burroughs Wellcome), caffeine and sodium benzoate (Ferris), acetazolamide (Lederle), inulin (Kerfoot), and heparin (Evans).

Results

Effects of Protein Deficient Diet

Rats which had been on the protein deficient diet for 11 weeks were apathetic and smaller than the controls. Some had scrotal oedema. Faeces were well-formed but of soft consistency. The mean body weight was about half that of the controls (Table I); most of this difference was due to a loss of weight of the dieted rats rather than to a gain in weight of the controls. The loss of weight in the first 5 weeks averaged 60.2 ± 4.6 g., and in the last 5 weeks 31.6 ± 5.1 g. The decreasing rate of weight loss may in part be due to the developing oedema (Haigh and Schnieden, 1956). Plasma protein and plasma albumin were reduced by the diet and the plasma volume was increased. There was a delayed bromsulphthalein excretion but the thymol turbidity test was normal. Plasma sodium was not changed. Water diuresis decreased as the diet was maintained, the mean time for 50% excretion of the water load increasing from 63 min. initially to 84, 116 and 171 min. after 5, 8, and 11 weeks on the diet. This was not due to a delay in water absorption from the gastro-intestinal tract, but there may have been a small delay in absorption from the gut itself (Table II). The main reason for failing water diuresis was a decrease in the glomerular

TABLE I

EFFECT ON ADULT RATS OF FEEDING A PROTEIN DEFICIENT DIET FOR ELEVEN WEEKS

The values given are for the mean ± S.E. Numerals in brackets are the numbers of animals in each group.

	High Protein Diet	Protein Deficient Diet
Body weight (g.)	210 ± 2 (24)	110 ± 9 (24)
Total plasma protein (g./100 ml.)	6·2 ± 0·6 (10)	4·3 ± 0·1 (21)
Plasma albumin (g./100 ml.)	4·3 ± 0·1 (8)	2·5 ± 0·2 (19)
Thymol turbidity test (units)	0–4 (12)	0–4 (12)
Bromsulphthalein test (% retention)	5·7 ± 0·5 (9)	39·7 ± 4·0 (10)
Haematocrit (% R.B.C.)	41·1 ± 4·0 (34)	28·5 ± 1·9 (20)
Evans blue space (ml./100 g.)	4·0 ± 0·1 (16)	6·5 ± 0·5 (10)
Blood volume (ml./100 g.)	6·8 ± 0·1 (16)	7·4 ± 0·2 (5)
Plasma sodium (m.eq./l.)	146 ± 1·4 (13)	145 ± 3·1 (12)

TABLE II

GASTRO-INTESTINAL WATER ABSORPTION IN PROTEIN DEFICIENT RATS AND CONTROLS

The values given are % of water retained (mean ± S.E.). Numerals in brackets give the numbers of animals/group.

% of Dose Retained in:	Controls	Protein Deficient Rats	*t*	*P*
Stomach	6·2 ± 1·4 (12)	6·3 ± 1·6 (10)	0·1	> 0·9
Intestine	8·2 ± 1·4 (12)	18·2 ± 5·2 (10)	2·0	< 0·05
Gastro-intestinal tract	14·3 ± 1·7 (12)	24·5 ± 6·3 (10)	1·6	> 0·1

filtration rate, which fell from 0.82 ± 0.8 (7) to 0.32 ± 0.06 (7) ml./100 g./min. Animals on the diet also reduced their fluid intake, which fell from 15.8 ± 0.9 (6) to 4.1 ± 0.8 (6) ml./100 g./24 hr.

Actions of Corticotrophin and Cortisone on Protein Deficient Rats

Corticotrophin had no effect at doses of 2.5 U./100 g. twice daily for 3 days given subcutaneously. Cortisone acetate given twice daily by subcutaneous injection for 3 days had the effects shown in Figs. 1 and 2. At doses of 2.5 mg./100 g. cortisone greatly improved diuresis. This change was accompanied by an improvement in appetite (Fouts, 1943; Meiklejohn and Passmore, 1951; Trowell, Davies, and Dean, 1952; Heller and Blackmore, 1953; Thompson, 1954), the food intake rising from 3.8 ± 0.3 (12) to 5.7 ± 0.5 (12). In spite of this increased food intake, the animals lost weight.

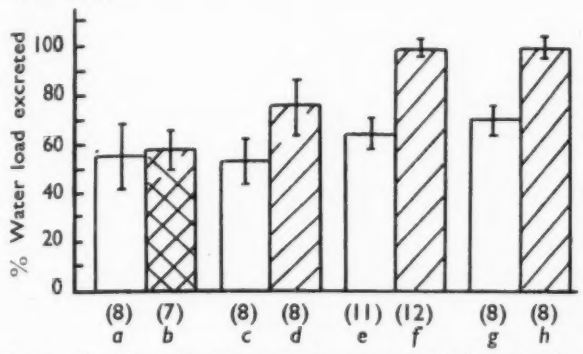

FIG. 1.—The effect of cortisone acetate on % water load excreted in 3 hr. (mean $\pm$ S.E.) by rats kept for 11 weeks on a protein deficient diet. (The numerals in brackets give the numbers of animals in group.) (*a*), (*c*), (*e*), and (*g*), control using cortisone suspending fluid. (*b*), control using 0.9% sodium chloride solution. (*d*), cortisone acetate, 1.25 mg./100 g. (*f*), cortisone acetate, 2.5 mg./100 g. (*h*), cortisone acetate, 3.75 mg./100 g.

The same dose of cortisone acetate increased the water intake of the protein deficient animals. The water intake of rats injected with cortisone suspending fluid for a period of 2 days was increased from 4.6 ± 0.5 to 7.1 ± 1.0 ml./100 g./24 hr.

There was an increase in inulin clearance but not in tubular reabsorption of water (Table III).

TABLE III

INULIN CLEARANCE AND TUBULAR REABSORPTION OF WATER IN PROTEIN DEFICIENT RATS TREATED WITH CORTISONE ACETATE OR WITH CORTISONE SUSPENDING MEDIUM

Values given are for mean $\pm$ S.E. Numerals in brackets are numbers of animals in each group. 2·5 mg./100 g. of cortisone acetate was given twice daily for 3 days. Tubular reabsorption of water = inulin clearance − urine flow in ml./100 g./min.

Treatment	Inulin Clearance		Tubular Reabsorption	
Injection with suspending medium	$0{\cdot}34 \pm 0{\cdot}07$ (14)	$t = 2{\cdot}3$ $P < 0{\cdot}02$	$83{\cdot}5 \pm 3{\cdot}7$ (6)	$t = 0{\cdot}8$ $P > 0{\cdot}4$
Injection with cortisone acetate	$0{\cdot}58 \pm 0{\cdot}08$ (12)		$84{\cdot}4 \pm 3{\cdot}2$ (6)	

Effect of Caffeine, Aminophylline, Acetazolamide, and Mersalyl on the Water Diuresis of Normal and Protein Deficient Rats

Normal Rats.—The results are shown in Table IV. Caffeine and sodium benzoate (10 mg./100 g.) were given twice daily intramuscularly for 3 days. No significant change in water diuresis resulted from this treatment. Acetazolamide was given once a day by stomach tube in a dose of 125 mg./100 g. At the end of the 180 min. period of observation % water load excreted by the rats treated with acetazolamide did not differ significantly from the controls, but there was a significant difference during the first 90 min. (% water load excreted by controls was 55.1 ± 4.3 (6)

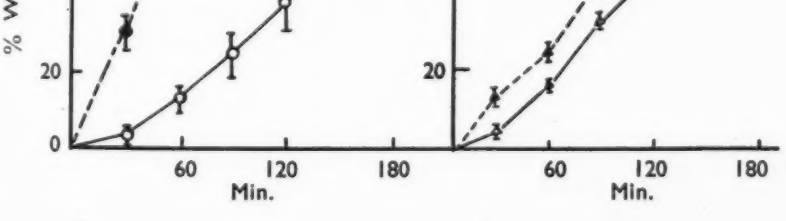

FIG. 2.—The effect of cortisone acetate (2.5 mg./100 g.) on the water diuresis of rats kept for 11 weeks on a protein deficient diet (mean $\pm$ S.E. of group of 12 rats). ○ Control before cortisone acetate. ● After cortisone acetate. △ Control before cortisone suspending fluid. ▲ Control with cortisone suspending fluid.

TABLE IV

EFFECT OF DIURETICS ON WATER EXCRETION OF NORMAL RATS

Numerals in brackets are numbers of animals in each group. Controls were injected with 0·9% saline. Treated rats were those receiving the diuretic.

Diuretic (for Doses see Text)	% Water Load Excreted in 3 Hr. by Normal Rats		t	P
	Treated Rats	Controls		
Caffeine and sodium benzoate	90·7±1·4 (6)	87·9±5·3 (6)	0·5	>0·5
Aminophylline ..	109·8±5·6 (6)	87·9±5·3 (6)	2·8	<0·01
Acetazolamide ..	93·4±1·6 (6)	87·9±5·3 (6)	1·0	>0·3
Mersalyl	63·1±3·7 (6)	95·0±5·7 (6)	3·4	<0·001

compared with 71.4±0.7 (6) for rats treated with acetazolamide: $t=3.7$; $P<0.001$). Aminophylline (4.0 mg./100 g. given intramuscularly twice daily for 3 days) had a significant diuretic effect. Mersalyl has been stated to exert its maximum diuretic effect in rats when given in a dose of 0.0027 ml./100 g. body weight 10 hr. before the diuresis test (Dicker, 1946). A single dose of mersalyl was therefore given intramuscularly following this procedure. There was no diuresis and in fact an antidiuretic effect was observed.

Protein Deficient Rats.—The same doses of all diuretics were used. Caffeine and sodium benzoate again failed to increase urine output. When a higher dose (16.7 mg./100 g. twice daily intramuscularly for 3 days) was used in a group of 7 animals, 3 died and the urine output of the remaining animals was similar to that of the controls. Neither acetazolamide nor aminophylline had any significant effect on water diuresis in the protein deficient rats. Mersalyl had again an antidiuretic effect (Table V).

TABLE V

EFFECT OF DIURETICS ON WATER EXCRETION OF RATS KEPT ELEVEN WEEKS ON A PROTEIN DEFICIENT DIET

Numerals in brackets are numbers of animals in each group. Treated rats received a diuretic. Control rats received (*a*), 0·9% sodium chloride solution; (*b*), cortisone suspending fluid; and (*c*), an injection contaminated with posterior pituitary gland hormones.

Diuretic (for Doses see Text)	% Water Load Excreted in 3 Hr. by Protein Deficient Rats		t	P
	Treated Rats	Controls		
Caffeine and sodium benzoate	54·6± 5·7 (10)	(*a*) 53·8±5·3 (9)	0·1	>0·9
Aminophylline ..	86·4± 6·8 (11)	(*a*) 74·4±9·3 (12)	1·0	>0·3
Acetazolamide ..	64·7±10·0 (12)	(*a*) 74·4±9·5 (12)	0·8	>0·4
Mersalyl	33·4±13·1 (12)	(*a*) 65·2±8·1 (10)	4·4	<0·001
Cortisone acetate ..	97·0± 7·0 (12)	(*b*) 65·4±6·4 (12)	3·6	<0·001
Corticotrophin ..	13·0± 2·9 (12)	(*c*) 50·6±9·4 (12)	4·3	<0·001

DISCUSSION

If we assume that the main reason for the impaired water diuresis of protein deficient rats is the observed decrease in glomerular filtration rate, an effect also noted by Dicker (1950) under similar conditions, diuretics like mersalyl and acetazolamide, acting exclusively or mainly on the tubules, would not be expected to produce a diuretic effect. However, xanthine diuretics such as aminophylline which has been reported to influence blood flow in the rat kidney (Dicker, 1946) also failed to increase water diuresis in our protein deficient animals. In contrast to this failure with the more conventional diuretics are the results with cortisone which raised the water diuresis of the protein deficient rats to levels well comparable to that of controls which had been kept on a high protein diet. Cortisone is known to increase water diuresis in normal rats (Winter, 1952; Dexter and Stoner, 1952). It has also been shown to increase the glomerular filtration rate and renal blood flow in healthy human beings (Ingbar, Relman, Burrows, Kass, Sisson and Burnett, 1950; Levitt and Bader, 1951) and the glomerular filtration rate in nephrosis (Deming and Luetscher, 1950; Luetscher *et al.*, 1950). In view of our negative results with the xanthine diuretics (especially since aminophylline in the dose used had a diuretic effect in normal rats) the glomerular effect of cortisone is probably not the only factor involved in its action. Silber and Porter (1953) have shown that cortisone causes a decrease in carcass proteins in adrenalectomized protein deficient rats but an increase in liver and plasma protein. This increase in plasma proteins may be an important factor in the diuretic action of cortisone demonstrated in our experience, because in nephrosis and in hepatic cirrhosis, in which, as in protein deficiency, the plasma albumin concentration is low, intravenously administered albumin has a diuretic effect and increases glomerular filtration rate (Luetscher, Hall, and Kremer, 1949; Orloff, Welt, and Stowe, 1949; Chinard, Lauson, Eder, Greif, and Hiller, 1954; Patek, Mankin, Colcher, Lowell, and Earle, 1948). It may well be that a combination of the metabolic effects of cortisone and its action on renal

blood flow and glomerular filtration rate are needed to influence the impaired water diuresis in protein deficient animals.

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References

Ahmed, N. D., and Frazer, A. C. (1952). *Proc. int. Congr. Biochem.*, 344.

Chinard, F. P., Lauson, H. D., Eder, H. A., Greif, R. L., and Hiller, A. (1954). *J. clin. Invest.*, **33**, 621.

Deming, Q. B., and Luetscher, J. A., Jr. (1950). Ibid., **29**, 808.

Dexter, D., and Stoner, H. B. (1952). *J. Physiol.*, **118**, 486.

Dicker, S. E. (1946). *Brit. J. Pharmacol.*, **1**, 194.

—— (1950). *Biochem. J.*, **46**, 53.

—— and Heller, H. (1945). *J. Physiol.*, **103**, 449.

—— —— and Hewer, T. F. (1946). *Brit. J. exp. Path.*, **27**, 158.

Fouts, P. J. (1943). *J. Nutrition*, **25**, 217.

Ginsburg, M. (1954). *J. Endocr.*, **11**, 165.

—— and Heller, H. (1953). Ibid., **9**, 267.

Guggenheim, K., and Hegsted, D. M. (1953). *Amer. J. Physiol.*, **172**, 23.

Haigh, C. P., and Schnieden, H. (1956). *J. Physiol.*, **131**, 377.

Hawk, P. B., and Bergeim, O. (1942). *Practical Physiological Chemistry*, 11th ed. London: Churchill.

Heller, H., and Blackmore, K. E. (1953). *Brit. J. Nutrition*, **7**, 349.

Heller, H., and Dicker, S. E. (1947). *Proc. Roy. Soc. Med.*, **40**, 351.

—— and Smirk, F. (1932). *J. Physiol.*, **76**, 1.

Hiller, A. (1927). *Proc. Soc. exp. Biol., N.Y.*, **24**, 385.

Ingbar, S. J., Relman, A. S., Burrows, B. A., Kass, E. H., Sisson, J. H., and Burnett, C. H. (1950). *Endocrinology*, **41**, 1.

Leslie, S. H., and Ralli, E. P. (1947). Ibid., **41**, 1.

Levitt, M. F., and Bader, M. E. (1951). *Amer. J. Med.*, **11**, 711.

Luetscher, J. A., Deming, Q. B., Harvey, J., Lew, W., and Poo, L. J. (1950). *J. clin. Invest.*, **29**, 1576.

—— —— and Johnston, B. B. (1951). Ibid., **30**, 1930.

—— Hall, A. D., and Kremer, V. L. (1949). Ibid., **28**, 700.

McLagan, N. F. (1944). *Brit. J. exp. Path.*, **25**, 15.

Meiklejohn, A. P., and Passmore, R. (1951). *Ann. Rev. Med.*, **2**, 129.

Orloff, J., Welt, L. G., and Stowe, L. (1949). *J. clin. Invest.*, **28**, 802.

Patek, A. J., Jr., Mankin, H., Colcher, H., Lowell, A., and Earle, D. P., Jr. (1948). Ibid., **27**, 135.

Peters, J. P., and Van Slyke, D. P. (1932). *Quantitative Clinical Chemistry*, 1st ed., Vol. II, p. 684. London: Ballière, Tindall and Cox.

Schnieden, H., and Blackmore, E. K. (1955a). *Voeding*, **5**, 489.

—— —— (1955b). *Brit. J. Pharmacol.*, **10**, 45.

Schreiner, G. E. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 117.

Silber, R. H., and Porter, C. C. (1953). *Endocrinology*, **52**, 518.

Thompson, M. D. (1954). *E. Afr. med. J.*, **28**, 31.

Trowell, H. C., Davies, J. N. P., and Dean, R. F. A. (1952). *Brit. med. J.*, **2**, 798.

Winter, C. A. (1952). *Ciba Foundation Coll. Endocrinology*, **4**, 449.

Woodman, H. E. (1952). *Bull. Minist. Agric. Lond.*, No. 48.

Brit. J. Pharmacol. (1957), **12**, 284.

THE METABOLISM OF EXOGENOUS AND ENDOGENOUS ANTIDIURETIC HORMONE IN THE KIDNEY AND LIVER *IN VIVO*

BY

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(RECEIVED FEBRUARY 6, 1957)

Negligible antidiuretic activity (less than 0.17 mU./g.) was found in extracts of the kidneys either of unanaesthetized adult rats in normal water balance or of rats in whose blood a rise of the level of endogenous antidiuretic hormone had been induced by ether anaesthesia. Extracts of the livers of unanaesthetized rats had negligible antidiuretic activity (less than 0.06 mU./g.), but liver extracts from rats anaesthetized with ether showed antidiuretic effects equivalent to 0.74 ± 0.24 mU. Pitressin/g. liver. When Pitressin was injected intravenously into unanaesthetized rats, small amounts of antidiuretic activity were occasionally found in the livers and the kidneys of animals killed up to 3 min. after the injection but none in animals killed later. Some 3% of the antidiuretic activity of an injected dose of Pitressin was found in the urine and the "dead space" of the kidneys in rats decapitated 3 min. after the intravenous injection. When Pitressin was added to rat kidney homogenate and the mixture was incubated at 38°, only 0.75% of the initial antidiuretic activity was recovered after 30 min. and less than 0.40% after 60 min. Experiments with "glomerular" and "tubular" fractions of rat kidney indicated that the inactivation was essentially due to tubular tissue. It is suggested that, in the rat, the kidneys and perhaps the liver are not only sites of clearance of the antidiuretic hormone but also sites of irreversible inactivation.

Ginsburg and Heller (1953a) and Crawford and Pinkham (1954) showed that, after intravenous injection into intact rats, the antidiuretic activity of doses of Pitressin (Parke Davis) ranging from 5 to 100 mU./100 g. body weight disappeared rapidly from the circulation: 5 min. after the injection less than 5% of the injected antidiuretic activity could be recovered from the blood. Similar experiments were done by Dicker (1954), who infused rats with pitressin at rates ranging from 30 to 100 μU./min./100 g. In 4 of his animals, killed 2 to 3 min. after the end of the infusion, no antidiuretic activity could be demonstrated in the plasma; in two others the recovery was equivalent to about 1% of the infused dose. However, when pitressin was injected or infused into nephrectomized rats (Ginsburg and Heller, 1953a; Crawford and Pinkham, 1954; Dicker, 1954), it was found that the antidiuretic activity was "cleared" at a considerably lower rate, the recovery after 2 to 5 min. varying from 37 to 63% of the administered dose. Sham-nephrectomized rats (Crawford and Pinkham, 1954) or animals with both ureters tied (Dicker, 1954) cleared the antidiuretic hormone at about the same rate as intact rats. It seems therefore that the kidney of the rat plays an important rôle in the removal of antidiuretic hormone from the circulation. But it is not the only organ so concerned. Ginsburg and Heller (1953a) found also that considerable amounts of the antidiuretic principle were removed in the splanchnic vascular bed, and Ginsburg (1957) subsequently found that little or no pressor activity was cleared by the intestine, suggesting that the liver was another organ of vasopressin clearance. These experiments fail to give any information about the fate of the hormone *in* these organs. There are obviously several possibilities: the active peptide may be temporarily stored or reversibly bound in these tissues, it may be excreted into the urine or the bile, it may be irreversibly inactivated, or a combination of these processes may apply. To investigate these possibilities the antidiuretic potency of extracts of the kidneys and the liver was estimated after raising the amount of circulating hormone either by injection of pitressin or by induced increase of the endogenous antidiuretic hormone.

METHODS

Adult albino rats of the Wistar strain (200 to 250 g.) were used. They were kept on a diet containing 25.6 g. protein (crude) and 0.3 g. NaCl/100 g.

Aqueous Extracts.—In preliminary experiments, aqueous extracts of the kidneys or the liver were prepared. Each rat was decapitated and bled, and the kidneys or liver removed as quickly as possible. The organs were weighed, cut into small pieces, transferred immediately into a test-tube containing boiling Krebs-Ringer solution and boiled for 3 min. The tube was then cooled, and the contents quantitatively transferred into a glass homogenizer and made up to the required volume. The mixture was centrifuged at 2,500 rev./min. and the supernatant used for assay. Other extracts of kidneys and liver were prepared in the same way using 0.25% acetic acid in 0.8% NaCl solution instead of Krebs-Ringer.

However, these methods of extraction were abandoned after some time because liver extracts prepared in either manner often produced antidiuretic effects. A pronounced but transient increase of urine flow preceding the antidiuretic effect was also seen in some rats. Similar effects were obtained with aqueous extracts of kidney although they occurred more rarely. When such aqueous extracts were intravenously injected into rats anaesthetized with urethane, it was found that they regularly lowered the blood pressure. These depressor effects were quite marked, for example a volume of extract equivalent to 20 mg. of kidney (wet weight) lowered the blood pressure by 21 mm. Hg. They were abolished neither by atropine nor antihistamines and may have been responsible for the "unspecific" renal effects just described.

Acid Ethanol Extracts.—Procedures used by Vogt (1953) for the extraction of vasopressin from the hypothalamus and by Bisset and Walker (1954) for the extraction of oxytocic and antidiuretic activity from blood, which are based on the method of Melville (1937), were therefore modified as shown on Table I. The interval between decapitation of the animal and immersion of the liver or the kidneys in ice-cold acid ethanol was approximately 3 min. Shortly after this method of extraction was instituted it was found that extracts neutralized to *p*H 7.2 in one stage frequently produced undesirable renal effects similar to those produced by aqueous extracts. This was probably due to the fact that part of the precipitate which forms at *p*H 4 to 5 redissolves on further addition of NaOH solution. The neutralization was therefore done in two stages. First, the precipitate formed at *p*H 4 to 5 was removed by centrifugation and, second, the clear supernatant was then adjusted to *p*H 7.2 and dialysed as described by Bisset and Walker (1954). Flamephotometric control estimations showed that 30 min. of dialysis reduced the potassium concentration of such liver and kidney extracts to less than 0.1 mg./ml. The freezing point depressions of some of the extracts were determined. They were found to have been hypotonic to rat plasma (mean and S.E. of 8 extracts, 0.40 ± 0.07).

TABLE I

EXTRACTION OF VASOPRESSIN FROM TISSUES WITH ACID ETHANOL

Tissue ground in glass mortar with ice-cold acid ethanol (1 ml. conc. HCl/l. of 98% ethanol, 10 ml. of this mixture/g. tissue)

↓

Transferred to centrifuge bottle with 30 ml. ethanol/g. tissue and centrifuged at 1,500 rev./min. for 15 min.

↓ Supernatant decanted

↓ 10 ml. acid ethanol/g. added to residue, again centrifuged at 1,500 rev./min. for 15 min.

↓ Supernatant decanted

↓ Combined supernatants concentrated *in vacuo* at 48° almost to dryness

↓ Residue taken up with 0.45% NaCl solution

↓ NaOH solution added until heavy flocculent precipitate appears, centrifuged at 2,500 rev./min. for 30 min.

↓ Supernatant dialysed against running water for 30 min. and adjusted to *p*H 7.0 to 7.2

Preparation of Kidney Homogenates for in vitro *Experiments with Pitressin.*—Kidneys from decapitated and bled animals were homogenized in a Potter-Elvehjem homogenizer with 10 or 20 ml./g. of Krebs-Ringer phosphate buffer (*p*H = 7.4.). Having been incubated for a given time, one volume of homogenate was mixed with 12 volumes of ice-cold acid ethanol and extracted as described on Table I.

Glomerular and tubular fractions of rat kidney were prepared by differential centrifugation in Krebs-Ringer solution (Lowell, Greenspon, Krakower and Bain, 1953). All procedures necessary for fractionation (including centrifugation) were done in a "cold room" at about +5°. Microscopical inspection showed slight contamination of the glomerular fraction with tubular elements. In order to make comparable incubation experiments with the two fractions, their content of solids had to be determined. Three samples of 2 ml. of each fraction were put into tared weighing bottles, dried at 105° for 37 hr., transferred to a desiccator containing phosphorus pentoxide, weighed, kept a further 12 hr. in the desiccator and reweighed.

Antidiuretic Assay.—Antidiuretic potency was assayed by intravenous injection of the extracts into unanaesthetized rats following the technique of Ginsburg and Heller (1953b) with the modification that not only a jugular vein but also the bladder was cannulated on the day before the experiment. Urine volumes were read at 5 min. intervals. The water load was kept at 6 to 8% of the body weight. Except when expressly mentioned, each assay consisted of the intravenous injection of four doses, two of the standard and two of the test material. The ratio of high to low doses was usually two. The order of injections was varied at random. Whenever possible each extract was assayed simultaneously on several rats. The volumes injected were always the same for standard and unknown. The antidiuretic effect of each dose was calculated in terms of % antidiuresis

as described by Ginsburg and Heller (1953b) with the difference that 5 min. collecting periods were used.

Estimation of Antidiuretic Activity in Urine.—To make the collection of urine as complete as possible, a fine polythene tube was inserted through the cannula and the bladder washed three times with small volumes of warm 0.9% NaCl solution. Emptiness of the bladder was verified by post-mortem examination.

The action of extracts on the blood pressure was tested in rats anaesthetized with urethane as described by Dekanski (1952). Pitressin (Parke, Davis) was used as the standard preparation.

Results

Antidiuretic Assays of Kidney Extracts of Normal Rats.—Normal rats are defined as animals which had been allowed food and water up to the time they were decapitated. In most instances acid ethanol extracts of the kidneys of such animals, in doses equivalent to 20 mg. fresh kidney, did not influence the water diuresis of the assay rats. Occasionally an antidiuretic effect was observed which, in contrast to the action of pitressin in the same animals, disappeared on repeated injection (Fig. 1). Such transient effects were therefore disregarded in this and all following experiments and it was assumed that renal extract of "normal animals" contained no demonstrable amount of vasopressin (Table II). In terms of the liminal sensitivity of the assay animals both kidneys contained less than the equivalent of about 0.23 mU. pitressin or less than 0.17 mU./g. kidney.

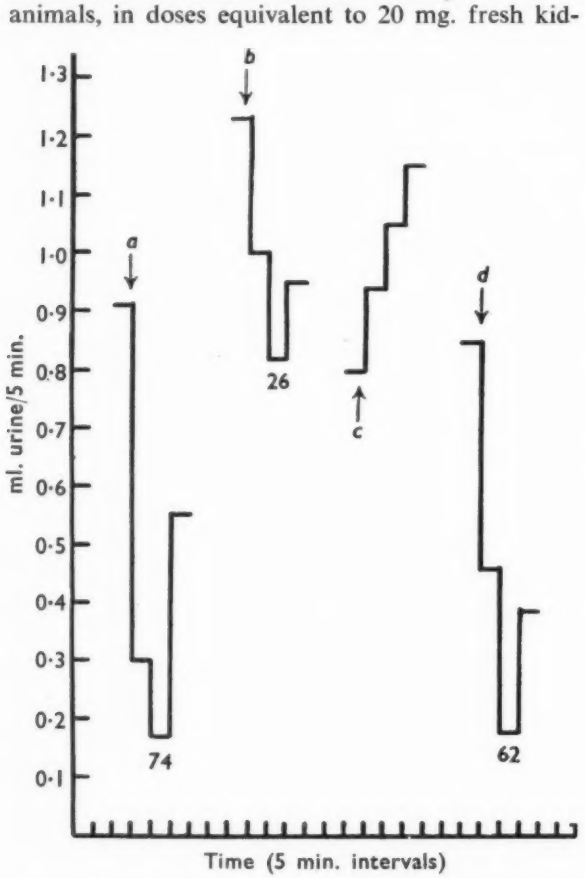

FIG. 1.—Transient antidiuretic effect of an acid ethanol kidney extract. Rat, 220 g. Intravenous injections. *a*, 100 μU. pitressin in 0.5 ml. of 0.9% NaCl solution. *b*, 0.1 ml. kidney extract (equivalent to 5 mg. wet weight of kidney) diluted to 0.5 ml. with 0.9% NaCl solution. *c*, 0.2 ml. kidney extract diluted to 0.5 ml. *d*, 50 μU. pitressin in 0.5 ml. of 0.9% NaCl solution. The numerals below each response indicate % antidiuresis.

TABLE II

ANTIDIURETIC ASSAYS OF ACID ETHANOL EXTRACTS OF KIDNEYS OF NORMAL RATS

Column 2 gives the dose of pitressin producing at least 25% inhibition of diuresis. The total volume of each extract (of both kidneys) was 30 ml.

No. of Extract (1)	Sensitivity of Rat (μU.Pitressin/Rat) (2)	Volume of Extract Injected Without Antidiuretic Effect (3)	Maximum Antidiuretic Activity in Both Kidneys in Terms of mU. Pitressin (4)
1	6·25	0·8	<0·23
2	6·25	0·8	<0·23
3	6·25	0 4	<0·46
4	6·25	0·3	<0·62
5	6·25	0·3	<0·62
6	6·25	0·3	<0·62
7	6·25	0·4	<0·46
8	6·25	0·4	<0·46
9	3·13	0·4	<0·23

TABLE III

ANTIDIURETIC ACTIVITY OF ACID ETHANOL EXTRACTS OF KIDNEYS FROM UNANAESTHETIZED RATS INJECTED WITH 100 mU. PITRESSIN/100 G.

Min. Killed After Injection	% Recovery of Injected Dose
1	<0·12, <0·12, <0·24, <0·24
3	0·20, 0·26, <0·12, <0·12
6	<0·45, <0·20, <0·45
12	<0·50, <0·25, <0·25
15	<0·50, 0·09

Antidiuretic Assays of Kidney Extracts of Rats Injected with Pitressin.—Table III shows the results of assays with renal extracts of unanaesthetized rats which had received intravenous injections of 100 mU. pitressin in 0.2 ml. saline/100 g. rat during 20 sec. It will be seen that antidiuretic activity was sometimes demonstrable in the renal extracts of animals killed up to 3 min. after the injection (counted from 10 sec. before the completion of the injection) but none in animals killed later. Similar results were obtained in rats anaesthetized with ether. The animals were anaesthetized for 10 min. and then decapitated. The standard dose of pitressin was injected during the 7th or 9th min. of anaesthesia.

No antidiuretic effects were obtained with kidney extracts of these animals. In two experiments the extract of both kidneys contained less than the equivalent of 0.45 mU. and 0.23 mU. pitressin respectively.

Before these results could be considered valid, it had to be shown that extracts of normal kidneys did not modify the action of added vasopressin, namely that they contained neither antagonistic nor potentiating substances. Fig. 2 (one of 9 similar experiments) shows that this did not apply. Furthermore, it was necessary to know how much of the antidiuretic activity of a dose of pitressin added to ground kidney could be recovered by our method of extraction. In 10 such experiments, in which 100 mU. was added to both finely divided kidneys, the mean recovery was $75.8 \pm 2.6\%$. Other recovery experiments were done by injecting 40 mU. pitressin contained in 0.02 ml. of 0.9% NaCl solution (the amount cleared by one kidney after the intravenous injection of 100 mU. pitressin/100 g. (Ginsburg and Heller, 1953a)) with an "Agla" micrometer syringe into the parenchyma of a kidney whose ureter was ligated immediately after decapitation. Recoveries in four such experiments were 86.0, 96.6, 68.4, and 100.0%. In three similar experiments, 5 mU. pitressin was injected into a kidney; the recoveries were 70.5, 55.0, and 70.0%.

There are reasons (see below) for believing that the true recovery was higher than is indicated by these results. However, even with recovery as low as about 75% it is evident from the result, shown on Table III, that, after an intravenous injection of pitressin, little of the large amounts of antidiuretic hormone cleared by the kidneys (Ginsburg and Heller, 1953a; Crawford and Pinkham, 1954; Dicker, 1954) can be extracted from kidney tissue. Is the disappearance of the hormone due to inactivation, or to excretion into the urine, or to both?

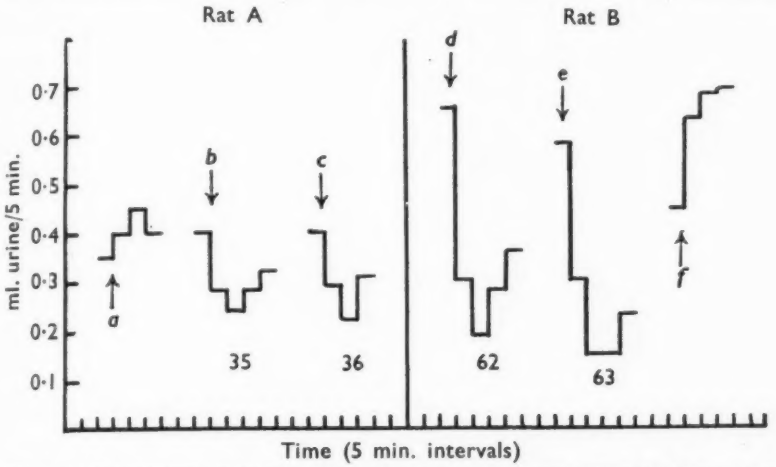

FIG. 2.—The antidiuretic effect of pitressin added to an acid ethanol extract of kidney. One ml. of extract was equivalent to 75 mg. wet weight of kidney. Intravenous injections. Rat A, 220 g. *a*, 0.4 ml. kidney extract. *b*, 0.4 ml. extract containing 6.25 μU. pitressin. *c*, 6.25 μU. pitressin in 0.4 ml. of 0.9% NaCl solution. Rat B, 270 g. *d*, 100 μU. pitressin in 0.3 ml. of 0.9% NaCl solution. *e*, 100 μU. pitressin in 0.3 ml. kidney extract. *f*, 0.3 ml. kidney extract. The numerals below each response indicate % antidiuresis.

Excretion of Antidiuretic Activity in the Urine.—The antidiuretic activity excreted by conscious rats in the first 3 min. after an injection of 100 mU./100 g. was estimated. All the urines, obtained by emptying and washing the bladder (9 experiments), inhibited the water diuresis of test animals. Assays were satisfactory in four of these experiments; the mean amounts excreted were equivalent to $0.70 \pm 0.17\%$ of the injected dose. Since the rate of urine flow in the donor rats was low and the urine collecting period very short, it is to be expected that antidiuretic activity would be present in the "dead space" of the renal pelves and the ureters which, in the experiments reported in Table III, had been carefully removed. Unanaesthetized rats were therefore injected with 100 mU. pitressin/100 g., killed after 3 min., the ureters tied close to the bladder and the kidneys removed together with the ureters. Extracts of such kidneys contained antidiuretic activity equivalent to $2.71 \pm 0.70\%$ of the injected dose, amounts which were much greater than those found in the parenchyma alone (Table III).

These results showed that about 3.4% of the antidiuretic activity of the injected pitressin was excreted by the kidney very rapidly within the first 3 min. after intravenous injection. However, it can be calculated from the results of Ginsburg and Heller (1953a) that the amounts of antidiuretic activity cleared by the kidneys of the rat in the same time (the same dose of pitressin was injected as in the present experiments) were 25% or more of the injected dose, suggesting that inactivation of the hormone in the kidney plays a more important rôle than renal excretion. Since little antidiuretic activity was recovered from the kidney parenchyma of rats killed 3 min. after injection and none from the kidneys of animals killed later (Table III) the rate of inactivation would appear to be high. Rapid inactivation may also partly

account for the incomplete recovery of antidiuretic hormone added to kidney tissue or injected into the kidney.

Antidiuretic Assays of Kidney Extracts of Rats Anaesthetized with Ether.—In order to obtain some information about the fate of endogenous vasopressin in rat kidney, the hormone level was raised by ether anaesthesia (Ginsburg and Heller, 1952, 1953c; Ames and van Dyke, 1952; Eversole and Giere, 1954) and the animals maintained in anaesthesia up to 10 min. No significant antidiuretic effects were obtained with kidney extracts of such animals.

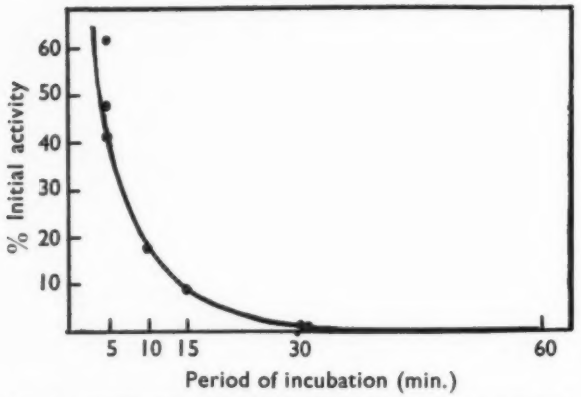

FIG. 3.—Inactivation of pitressin by rat kidney homogenate in Krebs-Ringer phosphate buffer (without Ca and Mg) incubated at 38°. Extraction by the acid ethanol method. (2+2) antidiuretic assays. For further details see text. Curve fitted by eye. At 60 min., the % activity remaining was <0.4%.

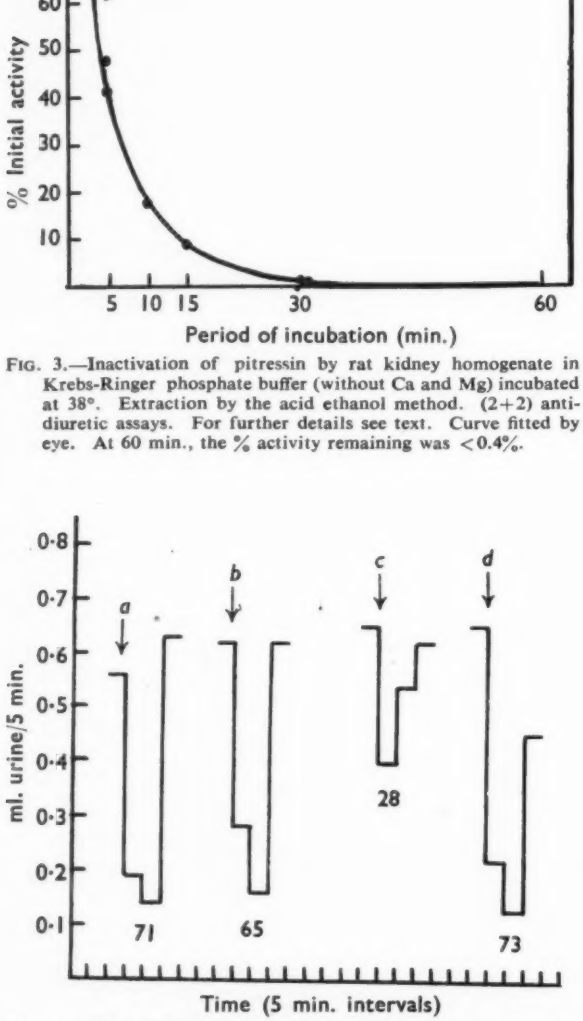

FIG. 4.—Effect of incubating pitressin with the glomerular and the tubular fractions of rat kidney for 30 min. at 38°. Antidiuretic assays. Intravenous injections of 0.4 ml./animal. Rat, 240 g. *a*, 200 μU. pitressin in glomerular fraction after incubation. *b*, 200 μU. pitressin in glomerular fraction before incubation. *c*, 220 μU. pitressin in tubular fraction after incubation. *d*, 220 μU. pitressin in tubular fractions before incubation. The amount of pitressin added to the fractions was proportional to the weight of suspended renal tissue. The numerals below the responses indicate % antidiuresis.

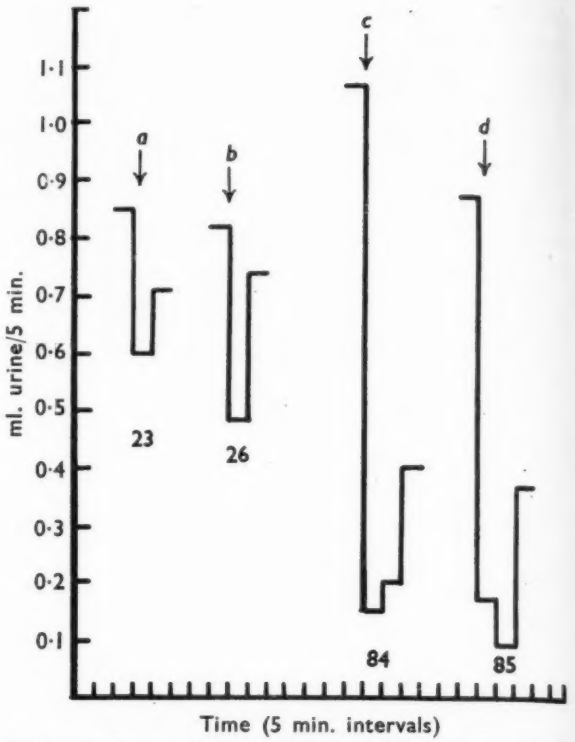

FIG. 5.—Incubation of arterial blood of the rat containing 10 units heparin and 1.0 mU. pitressin/ml. for 30 min. at 38°. Antidiuretic assay, intravenous injections. Rat, 108 g. *a*, 0.1 ml. of non-incubated blood diluted to 0.4 ml. with 0.9% NaCl solution. *b*, 0.1 ml. of incubated blood diluted to 0.4 ml. *c*, 0.3 ml. of non-incubated blood diluted to 0.4 ml. *d*, 0.3 ml. of incubated blood diluted to 0.4 ml. The numerals below the responses indicate % antidiuresis. The antidiuretic activity of incubated blood is 102.3% of that of the non-incubated mixture.

Inactivation of Vasopressin by Kidney Homogenates.—Our findings suggested that the kidney not only clears and excretes but also inactivates vasopressin *in vivo*. It seemed interesting therefore to study the rate of inactivation of vasopressin by kidney homogenates. Fig. 3 shows the rapid disappearance of antidiuretic activity in incubated mixtures of rat kidney homogenates and pitressin: 0.75% of the added activity remained after 30 min. of incubation and less than 0.4% after 60 min. These results are in good agreement with previously published results of one of us (Zaidi, 1955) and with those of Dicker and Greenbaum (1956) which were obtained with rat kidney homogenates from which aqueous extracts had been made. In an attempt to establish which renal tissue element was concerned in the inactiva-

tion of vasopressin, the hormone was incubated with homogenates of the glomerular and the tubular fractions of rat kidney. The effect of incubation of a dose of pitressin with the same amounts (in terms of solids) of glomerular and tubular homogenate is compared on Fig. 4. A substantial loss of activity was only obtained with the tubular fraction.

Inactivation of Vasopressin by Rat Blood.—It was possible that inactivation of vasopressin by kidney homogenate was partly due to blood trapped in the kidneys. However, Fig. 5 shows that incubation of vasopressin with heparinized rat blood for 30 min. caused no demonstrable loss of antidiuretic activity.

Antidiuretic Assays of Liver Extracts of Unanaesthetized Rats.—Since the liver may be another major site of vasopressin clearance in the rat, similar experiments were done with extracts of liver. Only slight and transient antidiuretic effects were seen with extracts of livers of normal animals (5 experiments). Expressed in terms of sensitivity of the assay animals (most of which responded to 3 μU. pitressin/100 g.), the antidiuretic activity was less than 0.44 mU./liver or less than 57 μU./g. liver.

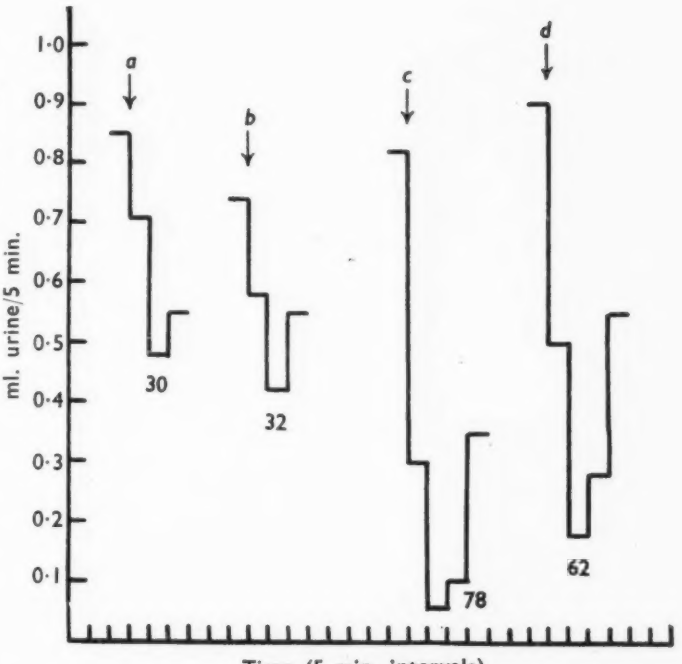

FIG. 7.—Recovery of endogenous antidiuretic activity in rat plasma added to rat liver suspension. Mixture immediately extracted by the acid ethanol method. The plasma concentration of antidiuretic hormone was raised by anaesthetizing the donor rat with ether. Antidiuretic assay. Intravenous injections. Recipient rat, 225 g. *a*, 0.2 ml. plasma. *b*, 0.2 ml. of extract of liver homogenate mixed with the plasma (1 ml. of extract was equivalent to 200 mg. wet weight of liver + 1 ml. plasma). *c*, 0.4 ml. plasma. *d*, 0.4 ml. extract The numerals below the responses indicate % antidiuresis. The recovery of antidiuretic activity from liver-plasma mixture was 88.4%.

Recovery of Vasopressin Added to Liver Extracts or Ground Liver Tissue.—Fig. 6 shows that the antidiuretic action of pitressin added to liver extract was neither enhanced nor diminished. When pitressin was added to finely divided rat liver and immediately extracted with acid ethanol, the mean recovery was 86.0 ± 3.4 (9)% of the added activity.

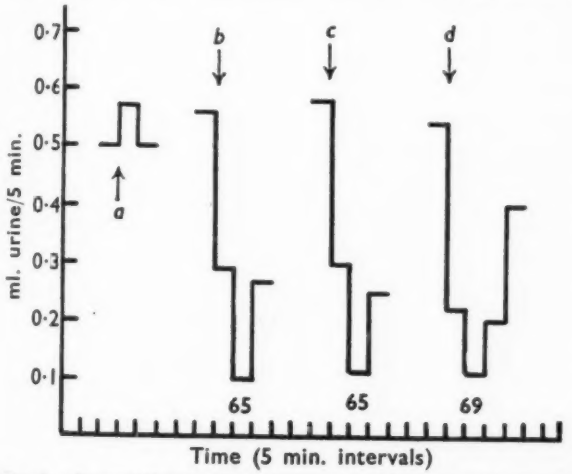

FIG. 6.—The antidiuretic effect of pitressin added to an acid ethanol extract of liver. One ml. of extract was equivalent to 200 mg. wet weight of liver. Rat, 235 g. Intravenous injections. *a*, 0.3 ml. liver extract. *b*, 50 μU. in 0.3 ml. of 0.8% NaCl solution, *c*, 50 μU. in 0.3 ml. liver extract. *d*, 50 μU. in 0.3 ml. of 0.9% NaCl solution. The numerals below the responses indicate % antidiuresis.

Antidiuretic Activity in Liver Extracts of Rats Anaesthetized with Ether.—In contrast to the results obtained in unanaesthetized animals, pronounced antidiuresis was produced by extracts of the liver of rats which had been kept under ether anaesthesia for 10 min. In 10 experiments the mean antidiuretic activity/liver was equivalent to 5.8 ± 0.8 mU. pitressin or to 0.74 ± 0.2 mU./g. liver, suggesting the possibility that ether anaesthesia interferes with the inactivation of endogenous vasopressin in the liver.

To ascertain whether the endogenous antidiuretic hormone could be recovered from liver tissue, the following experiment was done: The plasma of jugular blood enriched with endogenous vasopressin by subjecting the rat to ether anaesthesia was mixed with finely divided liver tissue obtained from an unanaesthetized animal; the

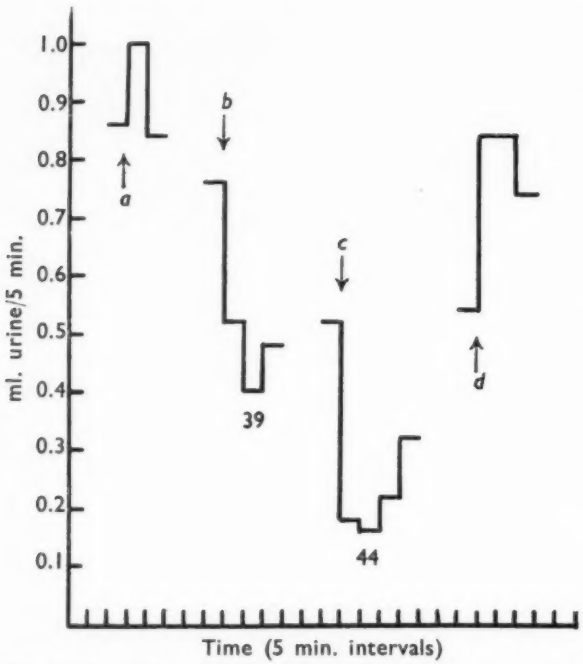

FIG. 8.—Comparison of the antidiuretic effect of liver extracts of hypophysectomized and intact rats. Both animals had been anaesthetized with ether for 10 min. and were then killed by decapitation. Antidiuretic assay. Intravenous injections. Recipient rat, 210 g. *a*, 0.4 ml. of liver extract of hypophysectomized rat. *b*, 100 μU. pitressin in 0.8 ml. of 0.9% NaCl solution. *c*, 0.8 ml. of liver extract of intact rat. *d*, 0.8 ml. of liver extract of hypophysectomized rat. The numerals below the responses indicate % antidiuresis.

mixture was immediately extracted with acid ethanol and the antidiuretic activity of the extract compared with that of the plasma. Fig. 7 shows a recovery of 88.4% of the "endogenous" antidiuretic activity from the liver extract. The recovery in another experiment was 92.2%.

The results are compatible with the possibility that the antidiuretic activity found in the livers of the anaesthetized animals was due to endogenous vasopressin. To test this assumption further, liver extracts were prepared from hypophysectomized rats which again had been anaesthetized with ether for 10 min. Only one of these experiments was entirely successful in that neurohypophysectomy in the donor was satisfactory and the recipient rats responded to sufficiently low doses of pitressin.

Fig. 8 shows that no antidiuretic effect was produced by the liver extract of the hypophysectomized and anaesthetized rat.

Attempts to study the inactivation of the antidiuretic factor in extracts of the livers of the anaesthetized animals by thioglycollic acid had to be abandoned, since intravenous injections of sodium thioglycollate itself occasionally produced an antidiuretic effect. The latter findings are contrary to the experience of Ames and van Dyke (1951) in rats anaesthetized with ethanol.

Antidiuretic Activity in Liver Extracts of Rats Injected with Pitressin.—Table IV shows that after the intravenous injection of 100 mU. pitressin/100 g. into unanaesthetized rats, substantial amounts of antidiuretic activity were recovered from the liver of the injected animal in one instance only.

TABLE IV

ANTIDIURETIC ACTIVITY OF ACID ETHANOL EXTRACTS OF LIVER FROM UNANAESTHETIZED RATS INJECTED WITH 100 mU. PITRESSIN/100 G.

Min. Killed After Injection	% Recovery of Injected Dose
1	<0·47, <0·46
3	0·61, <0·46, <0·43, <0·12, <0·92
6	<1·0, <0·97, <0·43, <0·48
9	<0·48
12	<1·0, <0·50
15	<0·52

DISCUSSION

Vasopressin has been shown to be inactivated by liver and kidney tissue *in vitro* (Heller and Urban, 1935; Jones and Schlapp, 1936; Larson, 1938; Christlieb, 1939; Eversole, Birnie, and Gaunt, 1949; Dicker and Greenbaum, 1954, 1956); but it is difficult to decide to what extent these results apply to the fate of the hormone *in vivo*. First, it seems likely that the rates of permeation and inactivation of the active principle will differ according to whether tissue homogenates or slices are used or whether the hormone circulates through an organ *in situ*. Secondly, cell injury in *in vitro* experiments may cause activation of enzymes or alternatively release enzyme inhibitors. Our results suggest that, *in vivo*, the kidney and liver inactivate vasopressin more rapidly than slices of these organs (Dicker and Greenbaum, 1954) and perhaps as rapidly as homogenates. We found only traces of antidiuretic activity in extracts of kidneys and livers of unanaesthetized rats decapitated 3 min. after the intravenous injection of pitressin, and no activity, demonstrable with our method of assay, was found in the organs of animals killed from 6 to 15 min. after the injection. However, some inactivation probably occurred also in the time taken (approximately 3 min.) to dissect the organs and to arrest enzymatic activity.

No vasopressin-like activity was found in the liver and kidneys of unanaesthetized rats in normal water balance. When rats were kept under

ether anaesthesia, killed by decapitation and bled, no antidiuretic activity could be demonstrated in the kidneys, but the liver contained substantial amounts of an antidiuretic substance. We cannot be certain that the latter was of neurohypophysial origin though the characteristics of the inhibitory effect and some results in hypophysectomized rats suggest it. Ether anaesthesia has been shown to increase the blood concentration of vasopressin (Ginsburg and Heller, 1952, 1953c; Ames and van Dyke, 1952; Eversole and Giere, 1954), but even if this raised blood level is taken into account the amounts of antidiuretic activity found were too large to have been due solely to the hormone content of the blood trapped in the liver. If ether anaesthesia interferes with the inactivation of the antidiuretic principle in the liver but not in the kidney, this would suggest that the mechanism of inactivation in the two organs is different.

In the rat, the kidneys play a threefold role in the metabolism of the antidiuretic hormone: (*a*) they remove a substantial fraction of the hormone from the blood (Ginsburg and Heller, 1953a; Crawford and Pinkham, 1954; Dicker, 1954), (*b*) they inactivate some of the cleared vasopressin, and (*c*) they excrete antidiuretic activity into the urine. So far as the process of inactivation is concerned there seem to be two possibilities: first, that vasopressin is transformed in the kidney to a less active metabolite which is then excreted, and secondly that some of the hormone escapes unchanged into the urine and that the unexcreted portion is completely inactivated. Dicker and Greenbaum (1954) suggested the first as the likely process, but recent experiments of Ginsburg, Heller, and Zaidi (1956) do not support this concept.

We are unable to say to what extent the results reported can be applied to the fate of "physiological" amounts of vasopressin in the body. The amounts injected in the present experiments or released by ether anaesthesia were large as compared with the very small doses needed to produce antidiuresis in the water-loaded rat. They are likely, for example, to have produced vascular changes which are not encountered when physiological amounts of the antidiuretic hormone are released.

It should be remembered, on the other hand, that, under pathological conditions like haemorrhage (Ginsburg and Heller, 1953c; Ginsburg and Brown, 1956), very large amounts of vasopressin may be released in the rat. Apparently such quantities can be inactivated with great rapidity.

The following question may be asked: if, as the present results suggest, a large dose like 100 mU./100 g. can be inactivated within the first few minutes after intravenous injection, why does the antidiuretic effect persist for 18 min. and longer (Ginsburg and Heller, 1953a)? Two answers may be given. First, since the plasma clearance of the antidiuretic hormone follows an exponential course (Ginsburg and Heller, 1953a), much smaller quantities of the hormone will be removed from the circulation (and subsequently inactivated) at low rather than at high plasma concentrations. The delay in reaching the minimum antidiuretic plasma level will therefore be considerable when a dose of the order of 100 mU./100 g. is injected. Secondly, changes in the effector cells may continue after the disappearance of the hormone from the blood and from the kidney.

Some similarity between the fate and metabolism of vasopressin and that of another active polypeptide, insulin, may be noted. The main sites for inactivation of insulin in the body, liver, and kidney (Weisberg, Friedman, and Levine, 1949; Haugaard, Vaughan, Haugaard, and Stadie, 1954; Elgee and Williams, 1954) are the same as those for vasopressin. The renal excretion of insulin, on the other hand, seems to be of less importance than that of antidiuretic hormone. The fate of other polypeptide hormones has not been sufficiently investigated to permit a comparison with that of vasopressin. However, the possibility of a common metabolic pathway of these active principles obviously deserves further study.

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References

Ames, R. G., and van Dyke, H. B. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 576.
—— —— (1952). *Endocrinology*, **50**, 350.
Bisset, G. W., and Walker, J. M. (1954). *J. Physiol.*, **126**, 588.
Christlieb, M. (1939). *Arch. exp. Path. Pharmak.*, **194**, 44.
Crawford, J. D., and Pinkham, B. (1954). *Endocrinology*, **55**, 699.
Dekanski, J. (1952). *Brit. J. Pharmacol.*, **7**, 567.
Dicker, S. E. (1954). *J. Physiol.*, **124**, 464.
—— and Greenbaum, A. L. (1954). Ibid., **126**, 116.
—— —— (1956). Ibid., **132**, 199.
Elgee, N. J., and Williams, R. H. (1954). *Proc. Soc. exp. Biol., N.Y.*, **87**, 352.
Eversole, W. J., Birnie, J. H., and Gaunt, R. (1949). *Endocrinology*, **45**, 378.

Eversole, W. J., and Giere, F. A. (1954). *Fed. Proc.*, **13**, 42.

Ginsburg, M. (1957). Cited by Heller, H., in *The Neurohypophysis*, Ed. Heller, H., *Proc. Colston Res. Soc. 8th Symposium*. London: Butterworth.

—— and Brown, Lucy M. (1956). *Brit. J. Pharmacol.*, **11**, 236.

—— and Heller, H. (1952). Cited by Heller, H., in *The Suprarenal Cortex*, Ed. Yoffey, J. M., *Proc. Colston Res. Soc. 5th Symposium*. London: Butterworth.

—— —— (1953a). *J. Endocr.*, **9**, 283.

—— —— (1953b). Ibid., **9**, 267.

—— —— (1953c). Ibid., **9**, 274.

Ginsburg, M., Heller, H., and Zaidi, S. M. A. (1956). *Nature, Lond.*, **178**, 803.

Haugaard, N., Vaughan, M., Haugaard, E. S., and Stadie, W. C. (1954). *J. biol. Chem.*, **208**, 549.

Heller, H., and Urban, F. F. (1935). *J. Physiol.*, **85**, 502.

Jones, A. M., and Schlapp, W. (1936). Ibid., **87**, 144.

Larson, E. (1938). *J. Pharmacol.*, **62**, 346.

Lowell, D. J., Greenspon, S. A., Krakower, C. A., and Bain, J. A. (1953). *Amer. J. Physiol.*, **172**, 709.

Melville, K. I. (1937). *J. exp. Med.*, **65**, 415.

Vogt, M. (1953). *Brit. J. Pharmacol.*, **8**, 193.

Weisberg, H. F., Friedman, A., and Levine, R. (1949). *Amer. J. Physiol.*, **158**, 332.

Zaidi, S. M. A. (1955). *J. Endocr.*, **12**, i.

Brit. J. Pharmacol. (1957), **12**, 293.

THE EFFECT OF INHALATION OF 30% CARBON DIOXIDE ON THE PERIPHERAL CIRCULATION OF THE HUMAN SUBJECT

BY

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(RECEIVED FEBRUARY 12, 1957)

Inhalation of 30% carbon dioxide for 1 to 2 min. caused a transient increase followed by a marked fall in blood flow through the normal forearm and calf. Since there was an associated large increase in arterial blood pressure, the fall in flow was due to intense vasoconstriction. It is likely that the vasoconstriction in the forearm occurred deep to the skin because the oxygen saturation of the superficial venous blood did not fall during the inhalation. Since there was a slight decrease in flow through the nerve-blocked forearm in spite of the increased blood pressure, it is unlikely that nervous vasoconstriction can completely account for the marked decrease in muscle flow.

The inhalation of 20 to 30% carbon dioxide in oxygen is occasionally used in the treatment of patients with nervous disorders (Meduna, 1948; Fay, 1953a and b). The present experiments describe some effects of 30% CO_2 on the cardiovascular system in two young men who were undergoing a course of such treatment for stammering.

METHODS

30% CO_2 in oxygen was administered from a cylinder connected to a large breathing bag and a close-fitting face mask. The subject lay on a couch in a room at 20 to 22° C. and breathed this mixture for 1 to 1½ min. (20 to 30 breaths) during each session. The respiratory movements were recorded by two stethographs, one around the chest and the other around the abdomen, connected to a single volume recorder. Forearm, hand and calf blood flow was measured by venous occlusion plethysmography using temperature-controlled plethysmographs (Greenfield, 1954). Digital heat flow was determined in some experiments with Hatfield (1950) heat flow discs fixed to the finger pulp. The fingers were immersed in water at 29° C. In one experiment on one of the subjects the radial, median and ulnar nerves of one side were infiltrated with local anaesthetic solution (Roddie, Shepherd, and Whelan, 1957a). Direct measurement of brachial arterial blood pressure was made with a capacitance manometer. The oxygen saturation of blood samples taken from a superficial forearm vein was determined by a spectrophotometric technique (Roddie, Shepherd, and Whelan, 1957b).

TABLE I

THE EFFECT OF 30% CO_2 ON FOREARM, CALF AND HAND BLOOD FLOW

The observations marked with an asterisk were made with plethysmograph temperatures of 44°.

Blood flow is expressed in ml./100 ml./min.

Subject	Blood Flow								
	Forearm			Calf			Hand		
	Before CO_2	During CO_2	Peak Flow after CO_2	Before CO_2	During CO_2	Peak Flow after CO_2	Before CO_2	During CO_2	Peak Flow after CO_2
O'N. ..	2·5	0·5	5·5	—	—	—	3·0	8·5	19·0
O'N. ..	5·0	1·0	12·0	4·0	1·5	9·0	—	—	—
O'N. ..	5·0	0·2	10·7	—	—	—	—	—	—
O'N. ..	4·0	0·2	13·0	4·2	0·5	10·0	—	—	—
O'N. ..	3·0	0·3	12·0	—	—	—	—	—	—
O'N. ..	—	—	—	3·0	0·4	11·5	2·0	2·0	10·0
O'N.* ..	13·5	13·0	19·0	—	—	—	21·0	30·0	38·0
M. ..	3·0	0·7	7·5	—	—	—	—	—	—
M. ..	6·5	3·6	12·0	—	—	—	11·0	6·5	24·0

RESULTS

Both subjects had had a previous course of treatment and did not appear apprehensive before the inhalation. During the inhalation there was a striking increase in the depth and to a lesser extent in the rate of respiration. Apart from the increased activity of the respiratory muscles there were no other involuntary muscular movements either during or after the inhalation and, though consciousness was dulled, it was not lost. The subject was sweating profusely at the end of the inhalation.

Forearm and Calf Blood Flow.—The forearm blood flow was measured at a plethysmograph temperature of 34° C. during inhalation of 30% CO_2 on eight occasions (Table I). There was an initial transient increase followed by a fall in blood flow which was maintained throughout the period of inhalation (Fig. 1). On some occasions the blood flow through the forearm almost ceased. When the inhalation of CO_2 was stopped a large transient increase in blood flow occurred. The changes in flow were found to be symmetrical on the two sides. Similar changes in flow were observed in the calf. The arterial pressure rose markedly, for example in one experiment from 125/75 to 205/110 (Fig. 1). Since the blood flow had decreased and the arterial blood pressure had increased, the resistance blood vessels of the forearm and calf must have been strongly constricted.

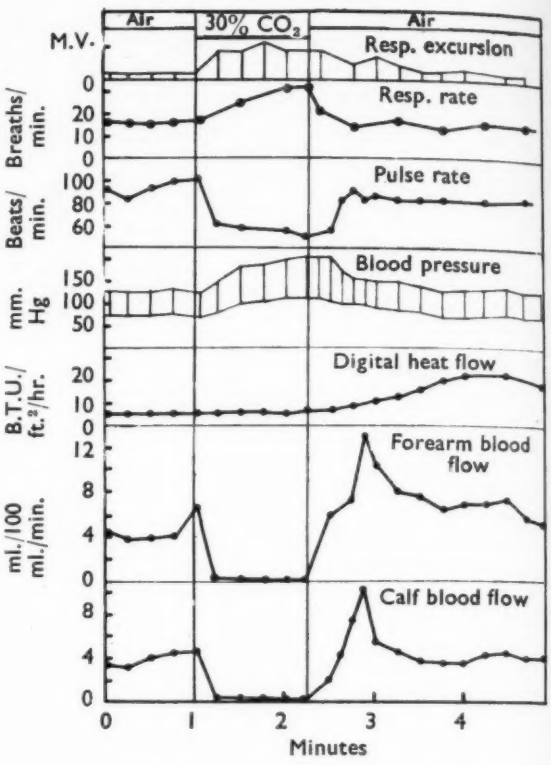

FIG. 1.—The effect of breathing 30% CO_2 in O_2 on respiratory excursion and rate, pulse rate, arterial blood pressure, digital heat flow and forearm and calf blood flow. The respiratory excursion obtained by maximal voluntary inspiration is designated M.V. Digital heat flow was measured by heat flow discs.

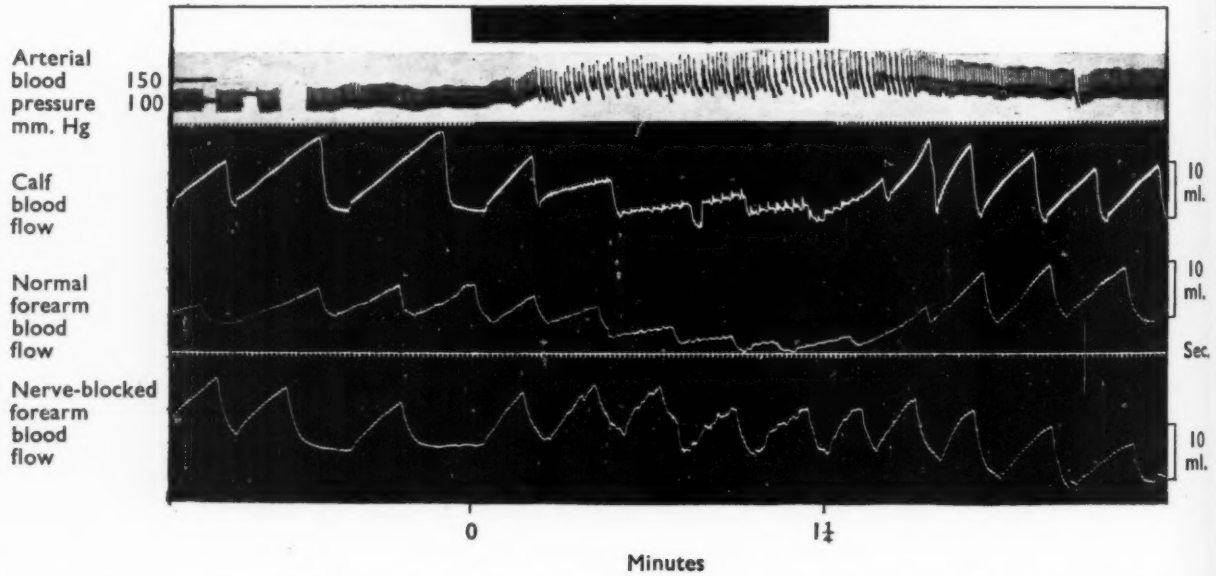

FIG. 2.—Arterial pressure and plethysmographic records obtained before, during, and after inhalation of 30% CO_2. The black rectangle represents the period in which CO_2 was administered.

Fig. 2 shows typical examples of plethysmograph and blood pressure records on one of the subjects.

In one experiment, the forearm blood flow on one side and the % oxygen saturation of superficial venous blood draining the skin of the opposite side were measured during CO_2 administration (Fig. 3). The circulation was arrested at both wrists by pneumatic cuffs inflated to 200 mm. Hg. During the CO_2 inhalation, the forearm blood flow decreased in the usual manner, but the oxygen saturation of the superficial venous blood increased from 80 to 91%. The increase in arterial blood oxygen content with 70% oxygen inhalation could at the most account for this increase. Even if it is assumed that it does so, it can be concluded that, provided the metabolism of the forearm skin remains constant, the blood flow through the skin is unchanged or possibly increased. The decrease in total forearm flow must be due to constriction of vessels deep to skin presumably in the skeletal muscles. On one occasion, with the plethysmograph temperature at 44° C., there was no change in forearm blood flow during the inhalation of CO_2. It is possible that under these conditions the increase in skin flow resulting from the rise in pressure combined with the dilatation of the skin vessels by the local heating was sufficient to balance the decrease in muscle flow.

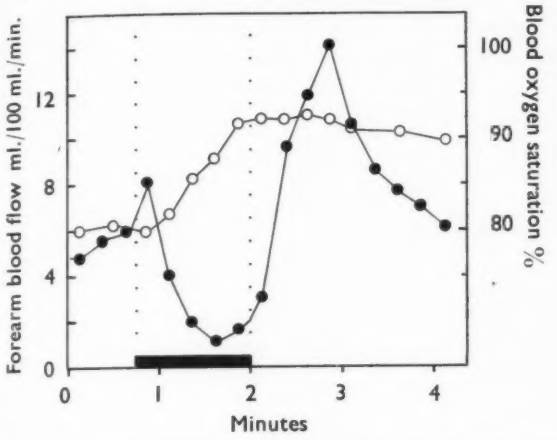

FIG. 3.—The effect of inhalation of 30% CO_2 for duration of black rectangle on forearm blood flow (●) and the oxygen saturation of the blood in a superficial forearm vein (○).

In one experiment (Figs. 2 and 4*a*) the flow through the normal and the nerve-blocked forearm was compared during CO_2 administration. There was the usual marked decrease in flow through the normal forearm and a small decrease through the nerve-blocked side. That the changes were not due to the mechanical effects of the hyperventilation is shown by the fact that hyper-

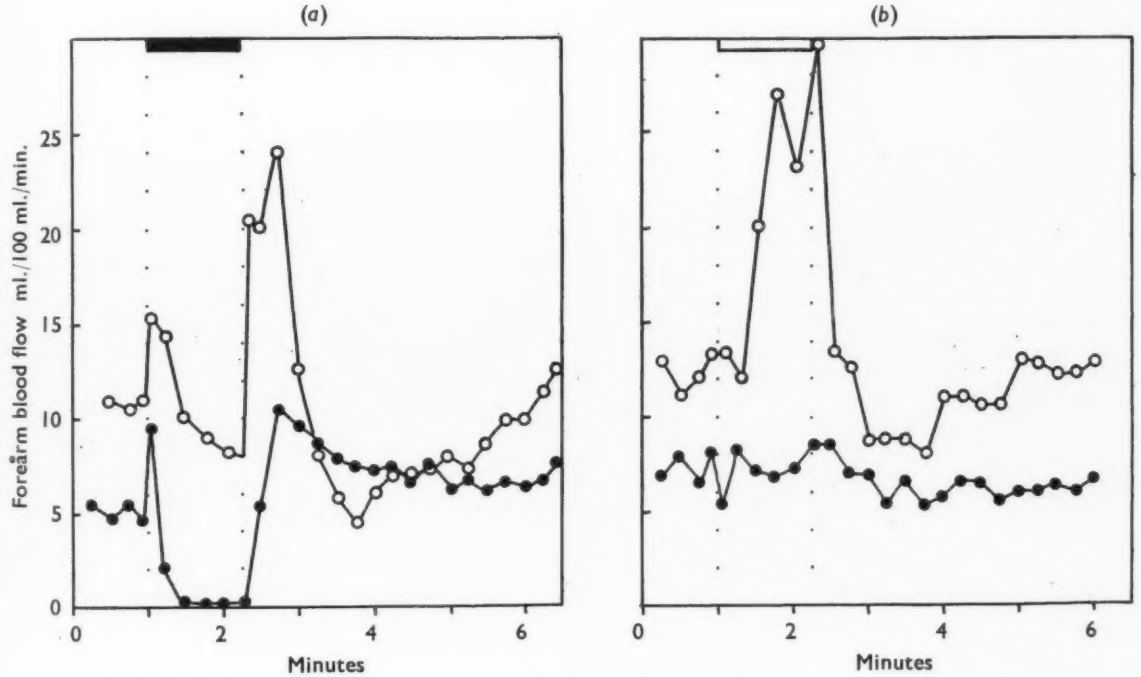

FIG. 4.—The blood flow through the normal (●) and nerve-blocked forearm (○). (*a*) During 30% CO_2 inhalation (black rectangle) and (*b*) during overbreathing air (clear rectangle).

ventilation in air at approximately the same rate and depth, which reduces the blood CO_2 level, produces a large increase in flow through the nerve-blocked forearm (Fig. 4*b*) (Roddie, Shepherd, and Whelan, 1957c).

Hand Blood Flow.—On three occasions the forearm and hand blood flows were measured simultaneously. Though the forearm blood flow always decreased, the hand blood flow on one occasion showed a transient fall, on another remained unchanged and on the third increased. Equivocal results were also obtained when digital heat flow was measured on two occasions. On one there was a fall in heat flow and on the other no change in flow during inhalation of 30% CO_2. As in the forearm, there was a transient increase in hand blood flow after the inhalation ceased. It is always difficult to distinguish changes in hand flow which result from emotional stimuli from changes due to other causes, but it seems that the response of these vessels was less marked and consistent than those of the forearm.

Discussion

It is generally believed that CO_2 by its action on either the vasomotor centre or the chemoreceptors can reflexly constrict peripheral blood vessels (Goodman and Gilman, 1955). Lennox and Gibbs (1932) found that breathing 4 to 8% CO_2 did not affect the blood flow through the leg and Abramson and Ferris (1944) found that, during inhalation of 7% CO_2, the forearm blood flow was either unchanged or increased. Abramson (1944) explained these findings on the basis that the blood vessels of the forearm are little, if at all, affected by vasoconstrictor impulses. Recent work, however, has shown that nervous control of skeletal muscle vessels plays an important part in the baroreceptor reflexes (Sharpey-Schafer, 1953; Roddie and Shepherd, 1956). While 30% CO_2 is outside the physiological range, the observation in the present experiments of intense constriction of normally innervated skeletal muscle vessels during inhalation of 30% CO_2 suggests that the sympathetic control of these vessels can play a part in the chemoreceptor reflexes.

It is unlikely that nervous vasoconstriction can completely account for the marked decrease in muscle flow since in the nerve-blocked forearm there was a slight decrease in flow in spite of the increased blood pressure. Failure to interrupt the sympathetic fibres by the nerve block is unlikely to explain these results; the forearm flow showed the characteristic increase after the block, the forearm and hand muscles were completely paralysed and there was a complete loss of sensation in the hand. Further, the increase in flow through this forearm with overbreathing room air was that typically seen in the denervated forearm (Fig. 4*b*).

During inhalation of 30% CO_2 there is a decrease in the arterial blood *p*H from 7.37 to 6.96 and the lactic acid changes from 8.0 to 8.75 mg. % (Gibbs and Gibbs, 1950). There is no evidence, however, that either of these changes would cause vasoconstriction. Liberation of noradrenaline could explain the decrease in flow through the nerve-blocked forearm as it has a direct constrictor action on muscle vessels. However, when given intravenously it often causes an increase in blood flow through the normal forearm due to an indirect dilator action (Barcroft, Gaskell, Shepherd, and Whelan, 1954).

It is most unlikely that there is a single explanation for the cardiovascular findings. Mechanical effects of the hyperventilation, emotional effects, the stimulation of chemoreceptors, the direct effect of carbon dioxide on the central nervous system, the chemical changes in the blood and the possible release of a humoral agent may all play a part in the final response.

References

Abramson, D. I. (1944). *Vascular Responses in the Extremities of Man in Health and Disease*, p. 228. Univ. of Chicago Press.

—— and Ferris, E. B. Cited by Abramson, D. I. (1944). Ibid., p. 227.

Barcroft, H., Gaskell, P., Shepherd, J. T., and Whelan, R. F. (1954). *J. Physiol.*, **123**, 443.

Fay, T. (1953a). *Amer. J. phys. Med.*, **32**, 338.

—— (1953b). *J. Amer. med. Ass.*, **152**, 1623.

Gibbs, F. A., and Gibbs, E. L. Cited by Meduna, L. J. (1950). *Carbon Dioxide Therapy*, p. 8. Illinois: C. C. Thomas.

Goodman, L. S., and Gilman, A. (1955). *The Pharmacological Basis of Therapeutics*, 2nd ed., p. 918. New York: Macmillan.

Greenfield, A. D. M. (1954). *J. Physiol.*, **123**, 62P.

Hatfield, H. S. (1950). Ibid., **111**, 10P.

Lennox, W. G., and Gibbs, E. L. (1932). *J. clin. Invest.*, **11**, 1155.

Meduna, L. J. (1948). *J. nerv. ment. Dis.*, **108**, 373.

Roddie, I. C., and Shepherd, J. T. (1956). *Clin. Sci.*, **15**, 433.

—— —— and Whelan, R. F. (1957a). Ibid., in the press.

—— —— —— (1957b). *J. clin. Path.*, **10**, 555.

—— —— —— (1957c). *J. Physiol.*, in the press.

Sharpey-Schafer, E. P. (1953). Ibid., **122**, 351.

Brit. J. Pharmacol. (1957), **12**, 297.

THE BLOCKING ACTION OF CHOLINE 2:6-XYLYL ETHER BROMIDE ON ADRENERGIC NERVES

BY

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Choline 2:6-xylyl ether bromide (TM 10), given systemically to cats in doses of 5 to 15 mg./kg., abolishes the effects of adrenergic nerve stimulation whilst leaving the reactions of the effector organs to adrenaline unimpaired. The effects of a single dose may take up to one hour to become fully established and last for more than twenty-four hours. Apart from transitory ganglionic blockade, cholinergic autonomic nerves are unaffected even by large doses of TM 10. Doses of TM 10 which produce effective blockade do not impair conduction along adrenergic nerve trunks; the drug must, therefore, act at, or close to, the nerve terminals. TM 10 prevents the output of noradrenaline from the spleen on stimulating the splenic nerves; but, in acute experiments, it does not influence the liberation of pressor amines from the stimulated suprarenals. Examination of some ethers related to TM 10 revealed no correlation between TM 10-like adrenergic blocking activity and local anaesthetic activity. The action of TM 10 on adrenergic nerves does not, therefore, seem to be accounted for by axonal block.

In 1954 Hey and Willey described the synthesis of choline 2:6-xylyl ether bromide (TM 10) and discovered that it abolished, for many hours, the effects of stimulating the postganglionic sympathetic supply to the nictitating membrane in cats, without affecting—except initially and transiently—the reactions to injected adrenaline.

Assuming that this paralysis might result from axonal block, they examined TM 10 for local anaesthetic activity when given intracutaneously to guinea-pigs, and found it to be a powerful and long-lasting local anaesthetic. The hypothesis of axonal block thus fitted the known facts. But whether conduction in postganglionic fibres was in fact abolished by doses of TM 10 which annulled the effects of stimulation was not directly tested.

The experiments described in this paper extend the observations of Hey and Willey to other nerves and effectors, and show that TM 10 does not abolish the effects of adrenergic nerve stimulation by preventing the passage of impulses along the nerve trunk, but by exerting an action at, or close to, the nerve terminals so that the impulses arriving there are rendered ineffective. A preliminary account of this work has already appeared (Exley, 1956).

METHODS

Experiments were done on the nictitating membrane, heart, pilomotor muscles, uterus, salivary glands, sweat glands, spleen, and suprarenals, in anaesthetized cats. Anaesthesia was induced with ether and maintained by chloralose (80 mg./kg., i.v.). Drugs were administered through a cannula in the femoral vein. Arterial blood pressure was recorded from a carotid artery by a mercury manometer. Some unanaesthetized cats and guinea-pigs were given TM 10 subcutaneously and their general reactions observed.

Electrical Stimulation.—Stimulation of nerve trunks was by rectangular pulses (0.5 msec. duration) applied through shielded electrodes. Contractions of the nictitating membrane were recorded in the usual way.

Nerve Action Potentials.—These were picked up through platinum electrodes and led into condenser-coupled amplifiers (time constant, 1 sec.). The output from these was displayed on oscilloscopes, one of which was fitted with a Cossor camera for recording purposes. The inferior cardiac nerve was prepared for stimulation close to the stellate ganglion in cats receiving artificial respiration and with the upper six ribs of the same side removed. All connexions to the stellate ganglion other than the cardiac branches were cut; action potentials were picked up from the distal part of the nerve, within 1 cm. of the heart.

The right cervical sympathetic trunk, with its superior cervical ganglion and postganglionic nerve, was exposed. The adjacent vagus nerve and nodose ganglion were excised. The preganglionic trunk was stimulated with either single or repetitive shocks, and biphasic action potentials in the postganglionic trunk were picked up by electrodes placed as far as possible from the ganglion. The ability of the nictitating mem-

brane of the same side to contract during a short period of tetanic stimulation at 10 shocks/sec. was checked from time to time. The tissues were covered with warm paraffin to prevent drying.

Heart Rate.—This was recorded on smoked paper by a Thorp electro-magnetic impulse counter operated by a relay and mercury switch. The latter was connected to the right ventricle by a hook and thread. The impulse counter was tripped to zero once every 10 sec. The thorax was opened and all nerve connexions to the right stellate ganglion were severed, except the cardiac branches which were laid on stimulating electrodes. The left vagus in the neck was cut and the distal trunk prepared for stimulation.

Uterine Movements.—The cervical end of one uterine horn of a non-pregnant cat was fixed *in situ*; the other end, after being detached from the ovary, was connected by a thread to a recording lever. The hypogastric nerves were ligated proximally and stimulated under warm paraffin.

Salivary Secretion.—The submaxillary duct was cannulated and the saliva allowed to displace saline, from a reservoir, into tubing connected to a Palmer Silver Drop Tube; a small amount of detergent was added to the saline to reduce the size of the drops, each of which operated an electro-magnetic marker. The chorda tympani nerve and the preganglionic cervical sympathetic trunk were prepared for stimulation.

Splenic Venous Blood.—This was obtained by the technique of Peart (1949), who showed that the transmitter of splenic nerve impulses was almost exclusively noradrenaline, a fact later confirmed by Mann and West (1950). Cats were pre-treated with atropine and had both adrenals ligated. Heparin (2,000 U./kg.) was administered after completing the dissection. Three-minute samples of blood, under the various control and experimental conditions, were collected into ice-cooled tubes containing heparin. The blood was immediately centrifuged; the plasma was drawn off, measured, and stored on ice until assayed. Between periods of collection, the venous blood was returned to the circulation through a polythene tube leading into the right femoral vein. The amine content of the plasma was assayed on the blood pressure of a cat which had been treated with atropine and ergotamine as described by Euler (1950). In later experiments, mepyramine maleate (5 mg., i.m.) also was given. The standard solution was of (−)-noradrenaline bitartrate, in terms of which the amine content of the plasma was estimated.

Results

Action of TM 10 on Cervical Sympathetic Nerves

Transmission to Nictitating Membrane.—The blocking action of TM 10 on the sympathetic supply to the nictitating membrane of the cat, discovered by Hey and Willey (1954), was readily confirmed. Fig. 1 shows a record of the contractions of the membrane during periodic postganglionic stimulation (15 sec. in each min., rate 50 shocks/sec.). Direct responses to adrenaline (10 μg., i.v.) were obtained before, and at various times after, the injection of 5 mg./kg. TM 10, the electrical stimulus being temporarily withheld during the adrenaline observations. The record shows the gradual development of the characteristic postganglionic block, accompanied, in the earliest stages, by a weak antisympathomimetic effect. The paralysis was persistent and never wore off during acute experiments such as this. Evidence will be presented later to show that the action of TM 10, though taking up to 1 hr. to become fully established, persists for 24 hr. or longer.

TM 10 also produced a failure of the nictitating membrane to respond to cervical sympathetic stimulation in rabbits anaesthetized with pentobarbitone.

Transmission to Iris.—In a cat not given atropine, a larger dose of TM 10 (10 mg./kg.) prevented dilatation of the iris on cervical sympathetic stimulation, though dilatation was readily obtained with intravenous injection of adrenaline.

Comparison of the Action of TM 10 on Adrenergic and Cholinergic Nerves

Though TM 10 is highly active in paralysing adrenergic nerves, the observation was made that the drug appears to exert no such effect upon postganglionic cholinergic nerves. This is illustrated by the following three types of experiment on animals not given atropine.

Actions on Parasympathetic and Sympathetic Nerves to the Heart.—The upper part of Fig. 2 shows the effects of 15 sec. periods of stimulation (50 shocks/sec.) to the cardio-accelerator nerve and to the left vagus, together with the effect of an intravenous dose of adrenaline. TM 10 was then administered; this caused an initial increase in heart rate, followed by a decrease which persisted throughout the experiment. (The drug invariably produced bradycardia in anaesthetized cats, whether atropine had been given or not.) The lower part of Fig. 2 shows the effects of nerve stimulation, and of adrenaline, some 20 min. after giving TM 10. The response to sympathetic stimulation was abolished whereas that to parasympathetic stimulation was unimpaired; the chronotropic effect of adrenaline appeared to be slightly augmented. Thus the effects of TM 10 were clearly confined to the sympathetic nerves to the heart: indeed, in similar experiments doses of TM 10 as high as 50 mg./kg. failed to produce sustained block of the vagus, though there was a

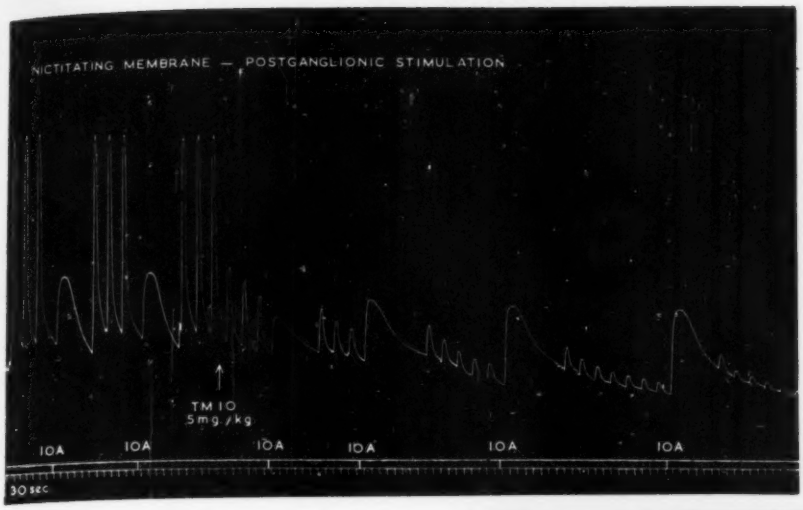

FIG. 1.—Cat, 2·1 kg., atropine, chloralose. Contractions of the nictitating membrane in response to postganglionic stimulation (15 sec. periods, 50 shocks/sec.) and to adrenaline (10 μg., i.v., at 10A) before and after TM 10 (5 mg./kg., i.v.). The onset of characteristic block to postganglionic nerve stimulation is seen after TM 10 with the transient anti-adrenaline action of the drug.

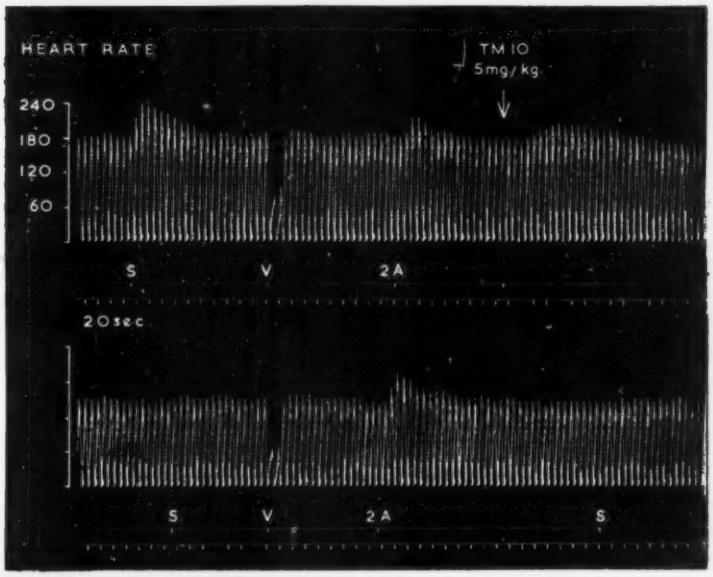

FIG. 2.—Cat, 3·7 kg., chloralose. Record of heart-rate response to stimulation (15 sec. periods, 50 shocks/sec.) of sympathetic (right cardio-accelerator, S) and parasympathetic (left vagus, V) nerves, and to adrenaline (2 μg., i.v., at 2A), before and after TM 10 (5 mg./kg., i.v.). Lower record started 20 min. after giving TM 10. The effect of sympathetic nerve stimulation is abolished by TM 10 while that of vagal stimulation remains. The response to adrenaline is not diminished.

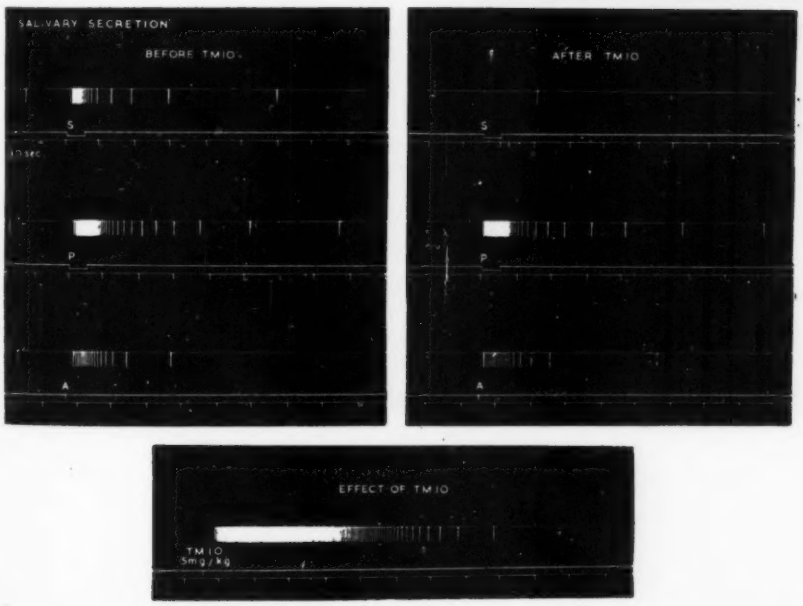

FIG. 3.—Cat, 2·1 kg., chloralose. Salivary secretion in response to stimulation (15 sec. periods, 50 shocks/sec.) of cervical sympathetic (S), or of chorda tympani (P), or to injection of adrenaline (10 μg., i.v., at A), before (upper left-hand records) and after (upper right-hand records) TM 10 (5 mg./kg., i.v.). The immediate effect of TM 10 is shown on the bottom record. The upper right-hand tracings began 20 min. after giving the TM 10. Each signal mark represents one drop of saliva. It is seen that only the response to sympathetic nerve stimulation is affected by TM 10.

paralysis for a few minutes after the injection, probably attributable in part to ganglionic blockade and in part to the weak antiparasympathomimetic action of the drug (Willey, 1957).

Actions on Parasympathetic and Sympathetic Nerves to Submaxillary Gland.—The effects of stimulation of these nerves, and of adrenaline, before and after 5 mg./kg. TM 10, are shown in Fig. 3. The normal responses are shown on the left, and those obtained 20 min. after TM 10 on the right. Again there was a clear-cut abolition of the sympathetic component without impairment of the effects of parasympathetic stimulation or of injected adrenaline. The injection of TM 10 itself evoked a brisk flow of saliva (lower block, Fig. 3). Further experiments showed that this secretion could be prevented by atropine, but not by hexamethonium. It is thus a further example of the muscarine-like action of TM 10, first noted by Willey (1957) on isolated guinea-pig ileum.

Actions on Sympathetic Nerves to Sweat Glands and Pilomotor Muscles.—It is well known that sweating is abolished by atropine, and Dale and Feldberg (1934) established the cholinergic nature of the sympathetic nerves supplying certain sweat glands in cats.

Following their technique, the caudal end of the cut left lumbar sympathetic chain was stimulated for periods of 30 sec. (50 shocks/sec.) at suitable intervals. Two responses were observed—the appearance of beads of sweat on the foot pads, and erection of the fur on the tail. Skin resistance in one foot pad was measured, and this showed an abrupt fall when sweating began. Twenty minutes after the injection of TM 10 (10 mg./kg.) the lumbar sympathetic was again stimulated; the pilomotor response was now absent, though sweating, with diminution in skin resistance, occurred exactly as before. Sweating was readily abolished, however, by a subsequent dose of atropine. Once more, therefore, it was evident that TM 10 was blocking adrenergic nerves, while leaving cholinergic nerves unaffected.

Effect of TM 10 on Action Potentials in Adrenergic Nerves

The fact that cholinergic postganglionic nerves were unaffected by TM 10 made it doubtful whether the characteristic action of the drug on adrenergic nerves could be ascribed to axonal block as such. In order to settle this point, therefore, the effect of TM 10 upon the action potentials picked up from three different adrenergic nerve trunks in the cat, the inferior cardiac, the splenic, and the postganglionic cervical sympathetic nerves, was investigated.

A series of three oscillograms of action potentials from the left inferior cardiac nerve, at a point within 1 cm. of its termination in the heart, are shown on the left of Fig. 4. The nerve was stimulated close to its exit from the stellate ganglion. The first potential was recorded before the drug was given, and the second 15 min. after the intravenous injection of 10 mg./kg. TM 10. The third was recorded 40 min. after giving the drug and was immediately preceded by a 30 sec. period of tetanic stimulation at a frequency of 50 shocks/sec. It is clear from these oscillograms that TM 10 did not depress the action potentials.

Exactly comparable results were obtained with the splenic nerves.

Oscillograms reproduced on the right of Fig. 4 were obtained from the postganglionic trunk of the

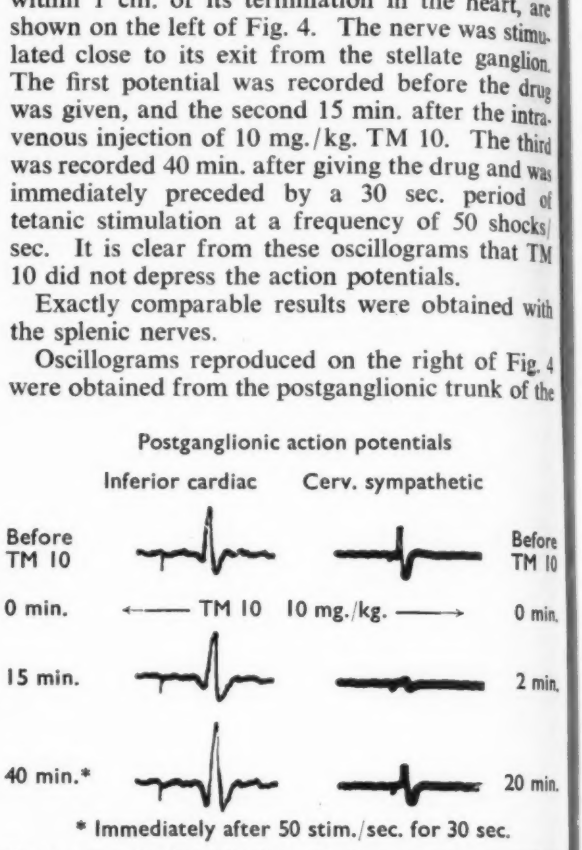

FIG. 4.—Cat, atropine, chloralose. Oscillograms of postganglionic action potentials from the inferior cardiac nerve (left) and from postganglionic fibres of the cervical sympathetic (right) following stimulation of the preganglionic trunk. TM 10 does not reduce the action potentials recorded from the inferior cardiac nerve, but it does produce a transient reduction in action potentials from the cervical sympathetic—probably because of ganglionic blockade. For further details, see text.

cervical sympathetic, and were evoked by preganglionic shocks. The upper oscillogram represents a normal biphasic action potential. Immediately after the intravenous injection of TM 10 (10 mg./kg.) there was an almost total suppression of the postganglionic action potential, as illustrated by the middle oscillogram taken 2 min. after giving the drug. This reduction was transitory and was probably due to a fleeting ganglionic blockade—as might be expected with a quaternary ammonium compound of this nature. Twenty minutes later the potential had returned almost to normal. But, at this stage, when the postganglionic potentials were passing normally along the nerve, preganglionic tetanic stimulation failed to evoke a response from the nictitating membrane.

It thus seems clear that the blocking action of TM 10 on the effects of adrenergic nerve stimulation cannot be attributed to a block of axonal conduction in adrenergic nerve trunks.

Action of TM 10 on Output of Pressor Amines

It is evident, since TM 10 does not impair the response of effector cells to adrenaline or noradrenaline, except immediately and transitorily, that it must reduce, in some way, the amount of mediator liberated by stimulated adrenergic nerves. It was of interest, therefore, to see whether this could be confirmed on a typical adrenergic nerve; and, if so, to determine if there was an analogous interference with the liberation of pressor amines from the stimulated suprarenal medulla.

Output of Noradrenaline from Spleen.—Samples of splenic venous blood, collected under various experimental conditions, were assayed for their pressor-amine content in terms of (−)-noradrenaline bitartrate. For the "post-stimulus" samples, the splenic nerves were stimulated (6 V., 50 shocks/sec.) during the first min. of a 3 min. collection period. Table I shows the results of one such experiment. The first two plasma samples were collected before the TM 10 was given. Stimulation of the nerves increased the total noradrenaline output/3 min., more than eightfold, and evoked a visible contraction of the splenic capsule. TM 10 (10 mg./kg. i.v.) was then given. A further resting sample (No. 3) was collected some 20 min. later. Subsequent stimulation of the splenic nerves now caused no detectable increase in amine output (Sample No. 4) over the resting level, nor did it evoke visible contraction of the splenic capsule. It was thus clear that TM 10 had prevented the liberation of noradrenaline in response to splenic nerve stimulation.

TABLE I

RESTING AND POST-STIMULATION NORADRENALINE CONTENTS OF SPLENIC VENOUS BLOOD PLASMA BEFORE AND AFTER TM 10

Sample No. and Description	Vol. of 3 min. Sample (ml. Plasma)	Noradrenaline Equivalent (μg./ml. Plasma)	Total Noradrenaline Output (μg./3 min.)
1. Resting	1·4	0·05	0·07
2. Post-stimulation	1·7	0·35	0·60
3. Resting, 20 min. after TM 10 (10 mg./kg.)	1·6	0·05	0·08
4. Post-stimulation, 25 min. after TM 10	1·6	0·05	0·08

Output of Amines from Suprarenals.—Experiments were done on the suprarenals, stimulated either through the splanchnic nerves or by chemical means.

For the splanchnic nerve experiments, cats were given atropine, anaesthetized with chloralose, eviscerated, and the kidneys tied off from the circulation. The distal portion of the cut left splanchnic nerve was stimulated for periods of 30 sec. (50 shocks/sec.). The resulting output of pressor amines was estimated on the arterial blood pressure and nictitating membrane of the same animal, by equating with suitable mixtures of (−)-adrenaline and (−)-noradrenaline given intravenously. The response of the nictitating membrane to stimulation of the preganglionic trunk of the cervical sympathetic was also tested occasionally.

Records of the arterial blood pressure, and of the contractions of the nictitating membrane, in such an experiment, are shown in Fig. 5. Before giving TM 10 the membrane responded fully to sympathetic nerve stimulation. The effects of splanchnic nerve stimulation were fairly accurately matched by a mixture of 2 μg. adrenaline and 3 μg. noradrenaline. One hour after TM 10 (5 mg./kg.) the response to cervical sympathetic stimulation was abolished; on the other hand, there appeared to be no change in the response to splanchnic stimulation. Larger doses of the drug (10 mg./kg.) similarly failed to modify the output of pressor amines on splanchnic stimulation.

In other experiments the suprarenals were stimulated by injections of tetramethylammonium bromide (TMA). Fig. 6 shows the response of the nictitating membrane of an atropine-treated cat to intravenous doses of adrenaline and of TMA, and to a lengthy period of postganglionic stimulation of the cervical sympathetic of the same side (10 shocks/sec.). The TMA caused a spiky contraction of the membrane, typical of ganglionic stimulation, followed by the familiar slow contraction owing to release of pressor amines from the suprarenals. TM 10 (5 mg./kg.) was given during the period of tetanic stimulation; the onset of nerve block is clearly demonstrated. Some time later the same doses of adrenaline and of TMA were repeated; the response to the former was not altered (lower half of Fig. 6), but that to TMA lacked the spiky ganglionic component. The slower component of the TMA response was still present, suggesting that the suprarenals were still discharging pressor amines. The suprarenals were then ligated, after which operation the response to TMA was abolished. A final electrical stimulus to the cervical sympathetic confirmed the persistence of a complete block.

The results of these experiments lead to the conclusion that TM 10 does not interfere, either qualitatively or quantitatively, with the release of

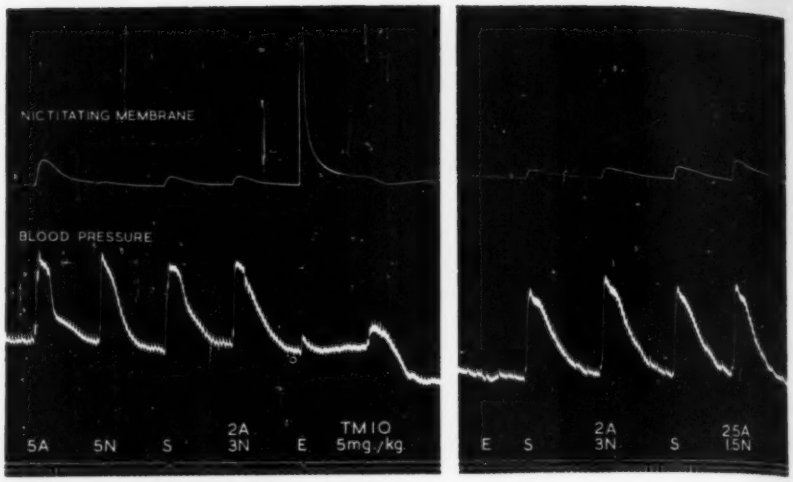

FIG. 5.—Cat, 2·8 kg., atropine, chloralose, eviscerated. Upper record, contractions of nictitating membrane; lower record, arterial blood pressure. Periods of left splanchnic nerve stimulation (30 sec., 50 shocks/sec.) at S and of cervical sympathetic stimulation (15 sec.) at E. Doses of adrenaline (A), noradrenaline (N), or mixtures containing the two amines were injected intravenously. The dose of each amine in μg. is indicated by the numerals preceding A or N. The record on right began 1 hr. after giving TM 10. Time, 30 sec. There is an abolition of effect of cervical sympathetic stimulation (E) after giving TM 10, while effect of splanchnic stimulation (S) persists.

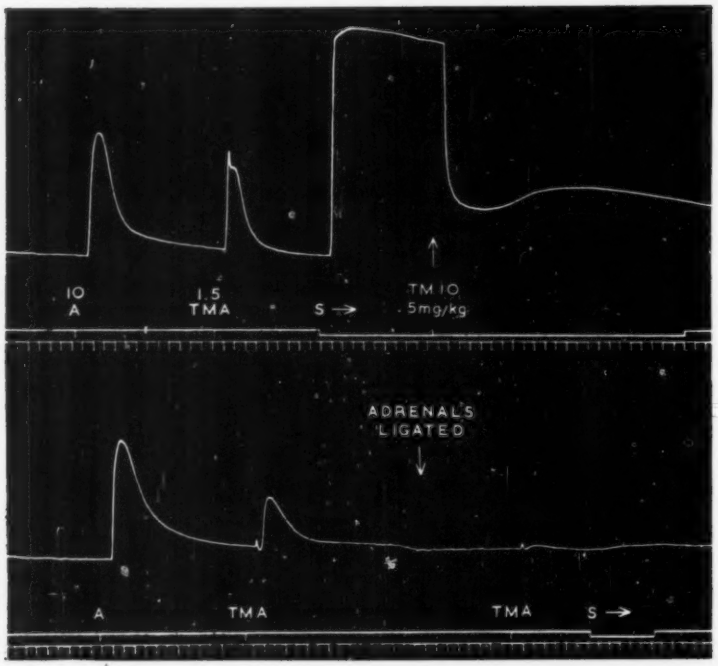

FIG. 6.—Cat, 2·9 kg., atropine, chloralose. Record of contractions of nictitating membrane evoked by 10 μg. adrenaline i.v. (10A), 1·5 mg. tetramethylammonium bromide i.v. (1·5 TMA), or by postganglionic stimulation of the cervical sympathetic at 10 shocks/sec. (S). Time, 30 sec. Lower record started 30 min. after TM 10 was given. For explanation, see text.

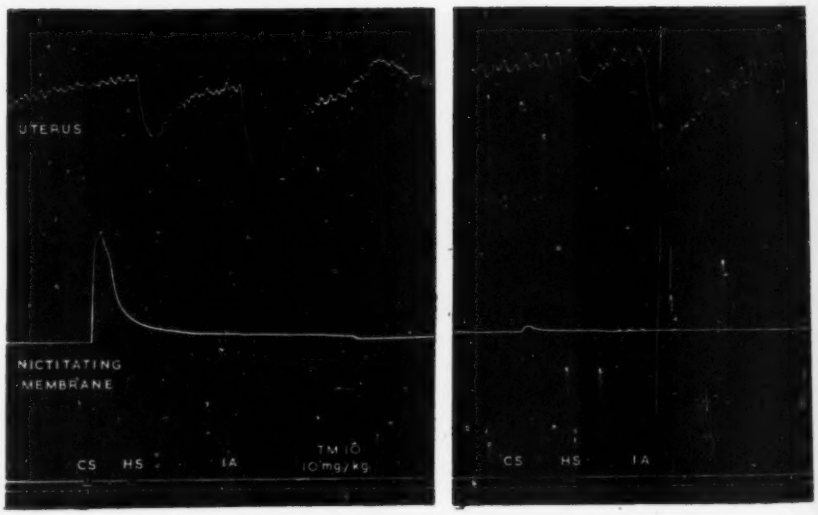

FIG. 7.—Cat, non-pregnant female, atropine, chloralose. Upper record, uterine tone; lower record, contractions of nictitating membrane. At 1A, adrenaline 1 μg. i.v. Stimulation (50 shocks/sec., for 30 sec.) of cervical sympathetic at CS, and of hypogastric nerves at HS. Time, 30 sec. Record on right started 1 hr. after TM 10. TM 10 greatly reduces the response of the uterus to hypogastric nerve stimulation while it appears to increase slightly the response to injected adrenaline.

pressor amines from the suprarenals stimulated under the conditions of these acute experiments. But experiments of this kind do not, of course, afford information about possible long-term disturbances within the gland. Such effects have been investigated, and will be reported separately.

Effect of TM 10 on Sympathetic Nerves to the Uterus

Dale (1906) first drew attention to the fact that the sympathetic nerves to the non-pregnant uterus exert an almost purely inhibitory action. As this inhibitory action, produced either by adrenaline or by nerve stimulation, is not readily blocked by antisympathomimetic drugs, it was of interest to see whether it would be affected by TM 10.

Such an experiment is illustrated in Fig. 7. Contractions of the nictitating membrane evoked by cervical sympathetic stimulation were recorded together with the relaxations of the uterus evoked by hypogastric nerve stimulation and by intravenous injections of adrenaline. One hour after giving TM 10 (10 mg./kg.) the responses to cervical sympathetic and to hypogastric nerve stimulation were greatly reduced, the former somewhat more than the latter. The sensitivity of the uterus to adrenaline was increased. In general, it has been found that hypogastric nerves are less readily blocked by TM 10 than are the other adrenergic nerves so far tested in cats, and as much as 15 mg./kg. of the drug may be required. On the other hand, the inhibitory effects of sympathetic nerve stimulation to the rabbit small intestine are readily abolished by TM 10 (Bain and Fielden, 1956).

Effects of TM 10 on Blood Pressure and Vasomotor Nerves

Doses of TM 10 sufficient to paralyse adrenergic nerve responses in cats tend to produce complex effects upon the blood pressure. The commonest pattern consists of a preliminary hypertension, developing slowly during the 5 min. following the injection, and lasting up to 30 min.; thereafter the blood pressure usually falls slowly to below its initial level. The nature of this preliminary hypertension has not been fully elucidated, but it is seen less frequently in animals whose adrenals have been ligated beforehand; the hypertension may occur in spinal cats and in animals treated with hexamethonium. It may be cut short by a dose of piperoxan (933F) or may be prevented from developing at all by a previous dose of this antisympathomimetic drug.

The vasomotor nerves appear to be paralysed by TM 10. As will be described later, the ears of unanaesthetized rabbits show a marked flushing after a systemic dose of the drug. Furthermore, the pressor responses, evoked in anaesthetized cats by stimulating the cut peripheral ends of the mesenteric nerves accompanying the superior mesenteric artery, are abolished by the usual doses of TM 10. In contradistinction, the pressor effect of splanchnic nerve stimulation may not be abolished by TM 10 even after the suprarenals have been ligated; the explanation for this may well be found in the large amount of extra-adrenal chromaffin tissue which cats possess. Such tissue is believed to be innervated by the splanchnic nerves, and would probably behave, after TM 10, in the same way as do the suprarenals themselves.

Effects of TM 10 on Unanaesthetized Animals

Hey and Willey (1954) investigated the toxicity of TM 10 when given intraperitoneally to mice. In cats, however, the drug appears to be poorly absorbed by this route. For the observations reported below, therefore, it was always given subcutaneously. Four guinea-pigs, given 20 mg./kg. TM 10, showed no outward changes in behaviour, or any immediate or delayed signs of prostration; there was, however, increased lachrymation for a short period after the drug was given. Much larger doses (60 mg./kg.) caused obvious muscular weakness.

Two cats were given 10 mg./kg. of TM 10 and observed during the ensuing 24 hr. Within an hour of the injection both animals had relaxed nictitating membranes and shivered; there were no signs of muscular weakness. Photographs of the eyes of one of these animals (*a*) before, and

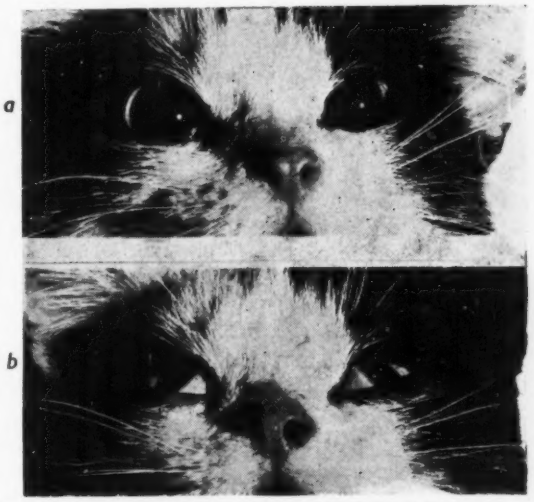

FIG. 8.—Eyes of unanaesthetized cat (*a*) before and (*b*) 24 hr. after a single dose of TM 10 (10 mg./kg., s.c.). The relaxation of the nictitating membranes is still present 24 hr. after the administration of TM 10.

(*b*) 24 hr. after the injection, when the effect on the nictitating membranes was still evident, are shown in Fig. 8. Forty-eight hours after giving the drug, partial recovery had occurred. One of the cats was then anaesthetized with chloralose; the response of the nictitating membrane to nerve stimulation was weak. On the other hand, the responses of the blood pressure and nictitating membrane to splanchnic nerve stimulation, after the animal had been eviscerated, were good, suggesting that the suprarenals were not affected.

The shivering produced by the drug in cats may result from increased heat loss, occasioned by cutaneous vasodilatation. Rectal temperature measurements in a rabbit confirmed that there was a fall in body temperature after TM 10, together with marked flushing of the ears.

TM 10-like Blocking Activity in Other Compounds

Hey and Willey (1954) made no mention of sympathetic-nerve blocking activity save in TM 10, though they found local anaesthetic properties in several related compounds. Some of the other ethers which they prepared were accordingly tested for TM 10-like activity.

The results, from experiments on the cervical sympathetic-nictitating membrane, can be summarized as follows: The *o*-tolyl homologue (choline 2-tolyl ether bromide, TM 18), seemed as active as TM 10 in producing the adrenergic-nerve block; but it has powerful nicotine-like stimulant properties and so the action could only be shown in animals given hexamethonium bromide (3 mg./kg.) and artificial respiration. The introduction of a third methyl group in the *para* position (choline 2:4:6-mesityl ether bromide, TM 17) markedly diminished activity.

With the cationic head of TM 10 altered to triethylammonium (β-(2:6-xylyloxy)ethyltriethylammonium bromide, TE 10), the specific blocking activity was lost, though, as reported by Hey and Willey (1954), this change did not destroy or impair the local anaesthetic activity. The tertiary analogues of TM 10 and TE 10 both had antisympathomimetic activity. But their action on the cervical sympathetic-nictitating membrane was brief, and could be fully accounted for by this antisympathomimetic action. There is no evidence that they interfere, as does TM 10, with the liberation of the transmitter. In their antisympathomimetic effects the tertiary amines (TTE 10 and TTM 10) are thus reminiscent of their parent phenyl homologues, whose properties have been described by Bovet and Maderni (1933) and by Justin-Besançon, Kohler, and Lévy (1937).

It can be concluded, therefore, that there is no direct relation between local anaesthetic activity in these compounds and TM 10-like adrenergic-nerve blocking activity.

Discussion

Hey and Willey (1954), who first made and investigated choline 2:6-xylyl ether bromide (TM 10), thought that its action upon postganglionic adrenergic nerves was sufficiently accounted for by the powerful and long-lasting local anaesthetic effect that they showed the drug to possess. The evidence presented in this paper, however, casts doubt upon the validity of this interpretation and raises the possibility that the drug may act upon adrenergic nerves in some other way. In particular, it has been demonstrated that conduction of impulses along three different adrenergic nerve trunks is not impaired by systemic doses of TM 10 sufficient to block the effect of adrenergic-nerve stimulation.

Furthermore, in parallel tests of the functional activity of both cholinergic and adrenergic nerves, such as those to the heart and salivary glands, cholinergic nerves are unaffected by doses of TM 10 that completely paralyse adrenergic nerves. Indeed, doses of TM 10 as high as 50 mg./kg. fail to block the effects of stimulation of the cardiac vagus nerve once the initial effects of interference with ganglionic transmission have worn off. If TM 10 were acting in virtue of its local anaesthetic properties, then postganglionic cholinergic fibres would be expected to show at least some vulnerability to the drug, especially fibres which run a long anatomical course, such as those to the sweat glands.

It might, of course, be argued that the local anaesthetic effect is not exercised on the main part of the sympathetic axon, but upon the delicate terminal fibrils which, being almost or completely devoid of myelin, might be expected to be specially vulnerable to circulating local anaesthetics, whether these be tertiary amines or quaternary ammonium compounds. But this would still fail to explain the immunity of pre- and postganglionic cholinergic nerve terminals to such an action—there being no reason to suppose that these are less susceptible to local anaesthetics than are the corresponding elements of adrenergic nerves.

Finally, quaternary compounds closely related to TM 10 may exhibit local anaesthetic properties equal to TM 10 itself (Hey and Willey, 1954), but possess, on systemic administration, little or no action upon adrenergic nerves. Thus the triethylammonium analogue (TE 10) is completely devoid

of the peculiar action of TM 10 on adrenergic nerves.

If, then, the adrenergic blockade produced by TM 10 is not connected with its local anaesthetic action it can result only from interference in some other way with the release or synthesis, or both, of the adrenergic mediators. Its action on adrenergic nerves may thus be analogous to that of botulinum toxin on cholinergic nerves.

My thanks are due to Professor W. A. Bain for his interest and valuable advice throughout this work, and for his help with the preparation of the text and figures; to Mr. R. Fielden for assistance with some of the experiments; and to Mr. D. Cameron and others for their technical help. The compounds used were synthesized by Dr. P. Hey.

References

Bain, W. A., and Fielden, R. (1956). *J. Physiol.*, **133**, 70P.

Bovet, D., and Maderni, P. (1933). *C. R. Soc. Biol., Paris*, **114**, 980.

Dale, H. H. (1906). *J. Physiol.*, **34**, 163.

—— and Feldberg, W. (1934). Ibid., **82**, 121.

Euler, U. S. v. (1950). *Acta physiol. scand.*, **19**, 207.

Exley, K. A. (1956). *J. Physiol.*, **133**, 70P.

Hey, P., and Willey, G. L. (1954). *Brit. J. Pharmacol.*, **9**, 471.

Justin-Besançon, L., Kohler, D., and Lévy, J. (1937). *Arch. int. Pharmacodyn.*, **55**, 282.

Mann, M., and West, G. B. (1950). *Brit. J. Pharmacol.*, **5**, 173.

Peart, W. S. (1949). *J. Physiol.*, **108**, 491.

Willey, G. L. (1957). *Brit. J. Pharmacol.*, **12**, 128.

Brit. J. Pharmacol. (1957), **12**, 306.

EFFECTS OF CHOLINE 2:6-XYLYL ETHER BROMIDE UPON THE SUPRARENAL MEDULLA OF THE RAT

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The administration of choline 2:6-xylyl ether bromide (TM 10) daily to rats for two weeks depletes the suprarenals of about one-half their normal content of adrenaline and noradrenaline. This depletion has been demonstrated histologically and by colorimetric and biological estimation of the amines present in extracts of the glands. Treatment with TM 10 causes similar histological signs of depletion in autografts of adrenal chromaffin tissue in the rat iris. Restoration of catechol amines in rat suprarenals, previously depleted by TM 10, occurs slowly and appears to be complete 14 days after withdrawing the drug. These results, considered in conjunction with the estimated rate of turnover of catechol amines in the rat suprarenal, lend support to the view that TM 10 may interfere with the biosynthesis of these amines.

Choline 2:6-xylyl ether bromide (TM 10) displays several pharmacological properties, including amine oxidase-inhibitory, ganglion-stimulant, muscarine-like, and neuromuscular-blocking actions (Brown and Hey, 1956; Willey, 1957; Edge, Mason, and Wyllie, 1957). Many of its actions are weak or transient, but one which is both interesting and pronounced is the ability of the compound to block the activity of adrenergic nerves for prolonged periods (Hey and Willey, 1954). Exley (1957) suggested that TM 10 may interfere, in some way, with the biosynthesis of the adrenergic nerve transmitter. He showed that the drug, given systemically, reduced the amount of transmitter liberated on stimulation of adrenergic nerves, but did not seem to impair conduction of impulses along such nerves; moreover, cholinergic postganglionic nerves were not similarly paralysed by TM 10. Doses sufficient to paralyse adrenergic nerves did not, in acute experiments, influence the liberation of pressor amines from the stimulated suprarenals of cats.

In a further attempt to elucidate the mode of action of TM 10, the effects of the chronic administration of this substance have been observed upon the normal rat adrenal medulla, and upon adrenal medullary grafts, using both pharmacological and histological methods.

The results suggest that the daily administration of TM 10 causes a progressive depletion of pressor amines both from the normally innervated gland, and from the non-innervated graft, such as might be expected as a result of either an interference with the biosynthesis of catechol amines or a direct stimulant effect on the chromaffin cells themselves.

METHODS

Male albino rats of Sprague-Dawley strain were used. In all experiments animals of comparable age and weight were chosen; many were litter-mates. All the rats were maintained under identical conditions of nutrition and temperature.

In preliminary experiments rats were given convulsive doses of insulin (10 units soluble/100 g. body weight, s.c.) and, after each animal had suffered three convulsions, sufficient amounts of 5% glucose saline were given subcutaneously to allow complete recovery. The insulin-treated animals were then divided into two groups, the animals in one receiving daily injections of TM 10 (10 mg./kg., s.c.), and the animals in the other receiving injections of isotonic saline. The adrenals were compared histologically after five days of treatment.

In a second experiment rats were given daily subcutaneous injections of TM 10 (10 mg./kg.), or of choline phenyl ether bromide (0.1 mg./kg.), or of an equivalent volume of isotonic saline. Some animals were killed after 7 days, and the remainder after 14 days. The adrenal glands were examined histologically. TM 10 (10 mg./kg./day) was also administered to 2 rats which had adrenal medullary autografts in the anterior chamber of the right eye. The autografts had been inserted three months previously by a technique described by Coupland (1955). One

animal was killed after 7 days and the other after 14 days of drug treatment; the autografts were examined histologically and compared with similar autografts taken from saline-treated rats.

In the final experiment two groups of rats were taken—one of 9 and the other of 6 animals. Each member of the larger group received daily injections of TM 10 (10 mg./kg., s.c.) for 14 days, while the other 6 animals (controls) received injections of isotonic saline. At the end of this time 7 of the TM 10-treated, and all of the saline-treated, animals were killed. The adrenal glands of one of the TM 10-treated animals were set aside for histology, and those of the others dissected free of fat, weighed, and extracted for pressor amines. The extracts were subsequently assayed, as were similar extracts made from the glands of the control rats. The two surviving members of the TM 10-treated group were killed 6 and 14 days after stopping the injections and their adrenals examined histologically.

Histological Methods

The adrenals were bisected with a razor-blade—one-half being fixed in formol-dichromate (namely, neutral formaldehyde 5%, potassium dichromate 3%) for 18 hr., mordanted in 2% potassium dichromate for 24 hr., washed in water for 24 hr., dehydrated and embedded in paraffin. Sections were cut at thicknesses of 6 and 8 μ. The other half of the gland was placed in saturated potassium iodate for 8 hr., and then transferred to 5% formaldehyde for 24 to 48 hr., after which frozen sections were cut at a thickness of 30 μ. The eye-grafts were fixed in formol-dichromate for 12 hr., mordanted in dichromate for 24 hr., washed, dehydrated, paraffin-embedded, and sectioned at 6 μ. All the paraffin-embedded sections were stained with haematoxylin or haematoxylin and eosin. All the tissues were processed in an identical fashion in order to avoid any variations in staining, etc.

Formol-dichromate fixation results in a staining of cells containing either adrenaline or noradrenaline; potassium iodate treatment results in a selective staining of those elements which contain noradrenaline (Hillarp and Hökfelt, 1954).

Assay of Pressor Amines

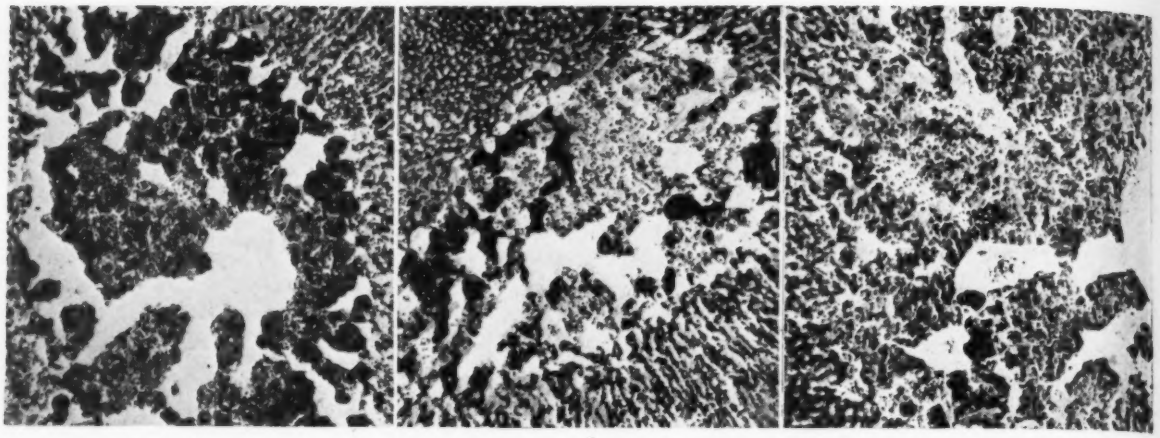
Both biological and colorimetric methods were used. The glands obtained from the TM 10-treated group of rats were pooled, weighed, and homogenized with 0.025 M-HCl, the *p*H adjusted to 5.5 by the addition of sodium acetate, placed in a boiling water bath for 3 min. and then cooled and centrifuged (Holland and Schümann, 1956). The adrenal glands of the saline-injected group were treated in the same way. The pressor amine content of the extracts obtained from the two groups of rats was estimated biologically by observing the effects on the blood pressure and acutely denervated nictitating membrane of a cat anaesthetized with chloralose and pre-treated (Coupland, 1953) with atropine sulphate (1 mg./kg., i.p.), cocaine HCl (5 mg./kg., i.m.), and mepyramine maleate (5 mg./kg., i.m.). Colorimetric estimations of the pressor amine content of the extracts were made by the method of Euler and Hamberg (1949).

RESULTS

Histological Observations

Effects of Insulin and TM 10 on the Adrenal Medulla.—The adrenals of rats killed five days after receiving convulsive doses of insulin were compared with those of rats similarly treated but given daily injections of TM 10, starting immediately after the insulin effects had subsided. Glands which were removed from animals five days after treatment with insulin alone showed a moderate chromaffin reaction (Fig. 1*a*) which was much greater than that observed in glands removed immediately after insulin treatment (Fig. 1*b*), thus indicating that some of the catechol amines lost were re-formed during the five-day period. The adrenal glands of rats treated with insulin followed by daily TM 10 for five days, on the other hand, showed only a faint chromaffin reaction (Fig. 1*c*). Thus the recovery of catechol amines appeared, from histological evidence, to be impeded by the daily administration of TM 10.

Comparison of the Effects of TM 10 and Choline Phenyl Ether Bromide on the Adrenal Medulla.—It was found, during the insulin experiments reported above, that TM 10 by itself was producing an effect on the chromaffin tissue. The effects of more prolonged administration of TM 10 upon the adrenals were therefore studied. Three groups of healthy rats were given daily injections of TM 10, isotonic saline, and choline phenyl ether bromide, respectively. The effects of choline phenyl ether bromide were studied, since it occurred to us that the action of TM 10 might be due to the weak nicotine-like stimulant properties of the drug. TM 10 has approximately 1/100th the stimulant activity of choline phenyl ether bromide (Willey, 1957) and so a daily dose of 0.1 mg./kg. of the latter drug was administered to the rats. Animals killed after seven days' treatment with TM 10 showed a moderately intense chromaffin reaction in the adrenals, and those killed after 14 days' treatment showed only a faint reaction (Fig. 2*a*). The chromaffin reactions of the choline phenyl ether- and saline-treated animals were, however, intense both at 7 and at 14 days (Fig. 2*b* and *c*). In all three groups of animals it was found that the staining properties of the noradrenaline-containing cells (as revealed by potassium iodate) were exactly similar to those of the whole medulla, described above—suggest-



a *b* *c*

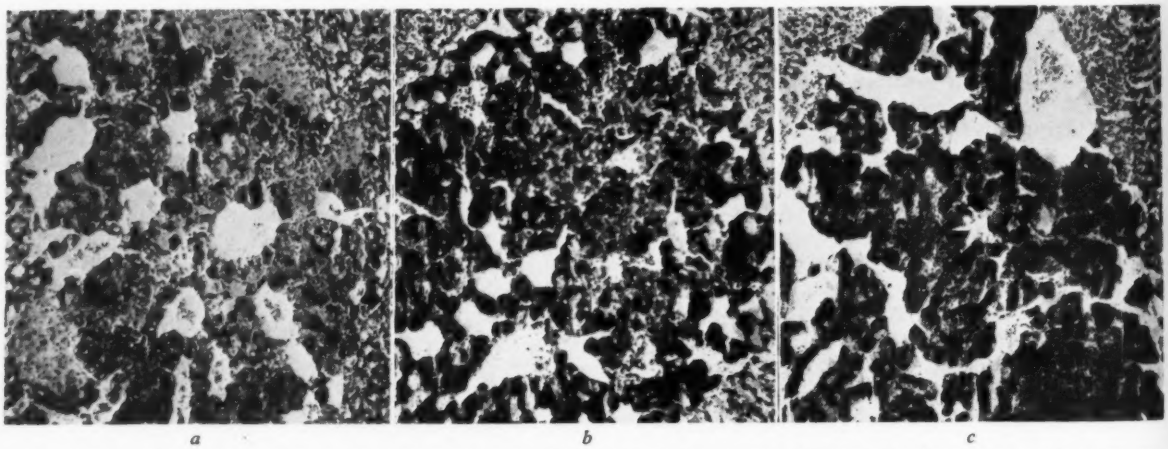
FIG. 1.—Medullary parts of rats' adrenals: (*a*) 5 days after receiving a convulsive dose of insulin; (*b*) immediately after three insulin convulsions; (*c*) 5 days after insulin convulsions and daily injections of TM 10. The restoration of chromaffin reaction after insulin shock is delayed by daily injections of TM 10. Formol-dichromate; haematoxylin; × 70.

ing that adrenaline and noradrenaline levels in the adrenal are equally influenced by TM 10.

These experiments suggest that TM 10 reduces the chromaffin reaction progressively, and by some other mechanism than nicotine-like stimulation of the medullary cells.

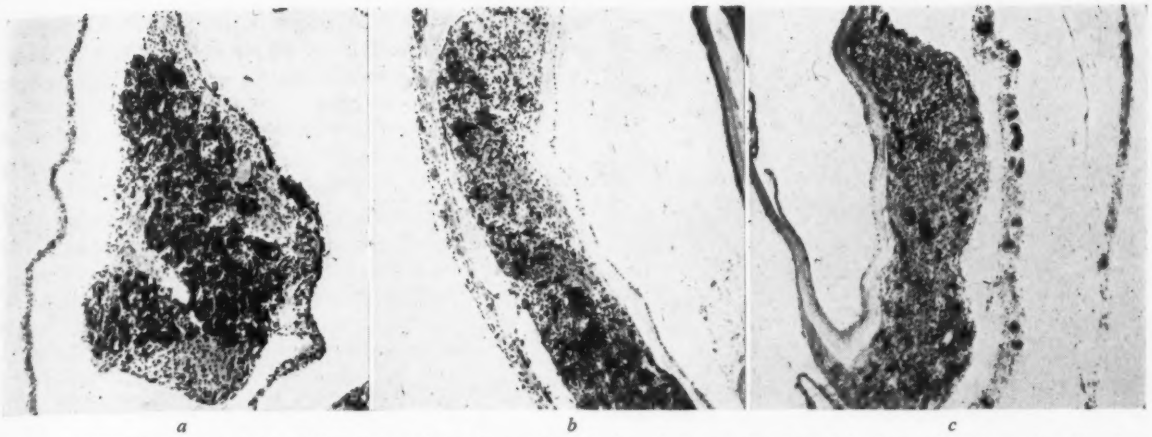
Effect of TM 10 on Autografts of Adrenal Medullary Tissue.—By comparison with control grafts (Fig. 3*a*), autografts obtained from rats treated with TM 10 showed a striking reduction in chromaffin reaction (Fig. 3*b* and *c*). The intensity of staining is less in the graft removed after 14 days' drug treatment than in the one removed after only seven days. Hence non-innervated, grafted, medullary cells appear to be affected by TM 10 in a similar way to those within the intact adrenal gland.

Changes in the Adrenal Medulla after Cessation of TM 10 Treatment.—Three rats, belonging to the group treated with TM 10 for 14 days (for the purposes of the quantitative experiment described below), were set aside for histological examination. One of these was killed one day after stopping the TM 10 injections; its adrenals showed, as expected, a very faint chromaffin reaction (Fig. 4*a*). Another animal was killed six days after stopping the treatment; its adrenals showed a moderate chromaffin reaction (Fig. 4*b*). The remaining rat was killed after 14 days and the chromaffin reaction was now indistinguishable from normal (Fig. 4*c*).



a *b* *c*

FIG. 2.—Rats' adrenal medullae after 14 daily injections of (*a*) TM 10, (*b*) choline phenyl ether bromide, (*c*) isotonic saline. Choline phenyl ether has little effect on the intensity of the chromaffin reaction, whereas TM 10 causes a marked reduction. Formol-dichromate; haematoxylin; × 70.



a *b* *c*

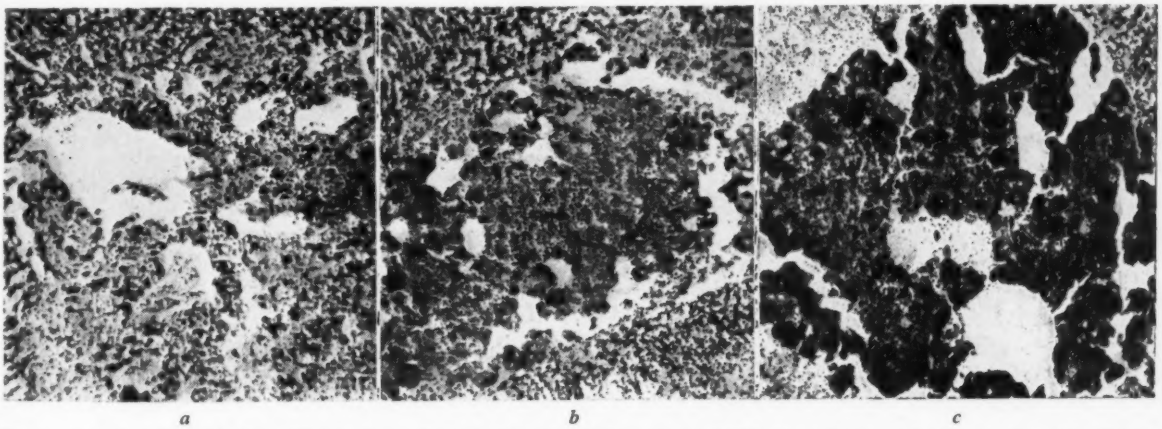
FIG. 3.—Autografts of rat adrenal medullae removed from the anterior chamber of the eye after daily injections of (*a*) isotonic saline for 14 days, (*b*) TM 10 for 7 days, (*c*) TM 10 for 14 days. TM 10 treatment reduces the intensity of the chromaffin reaction. The dark islands present in both graft and iris in (*c*) are intravascular collections of blood cells. Formol-dichromate; haematoxylin; ×70.

It is therefore concluded that, after TM 10 treatment under the conditions described, an interval of between 6 and 14 days is required for the restoration of the normal chromaffin-staining properties of the adrenal medulla.

Quantitative Estimation of the Degree of Depletion of Catechol Amines

Two groups, each of six rats, were used for the quantitative experiments. Animals in one of the groups were injected daily with TM 10 for 14 days (10 mg./kg., s.c.), whilst animals in the control group received daily injections of an equivalent volume of isotonic saline. At the completion of the treatment period the animals were killed, their adrenals removed, and the pooled weight of the glands determined for each group. For the TM 10-treated group, the total weight of the glands was 297 mg., whilst for the saline-treated group the weight was 285 mg.—weights close enough in agreement to exclude the occurrence of any significant atrophy or hypertrophy of the glands in rats receiving TM 10.

Extracts of the glands were made and the amounts of adrenaline and noradrenaline in each extract determined, biologically on the cat blood pressure and nictitating membrane, and colorimetrically, using solutions of pure (−)-adrenaline and (−)-noradrenaline, or mixtures of these, as standards of reference. Fig. 5 shows part of a record of the contractions of the nictitating membrane and blood pressure from the cat used for the estimation. The extract obtained from the TM 10-treated rats' adrenals contained only about



a *b* *c*

FIG. 4.—Rat adrenals removed at different times after cessation of a 14-day course of TM 10: (*a*) removed after 1 day; (*b*) after 6 days; (*c*) after 14 days. The chromaffin reaction returns slowly and is moderate after 6 days but intense at 14 days. Formol-dichromate; haematoxylin; × 70.

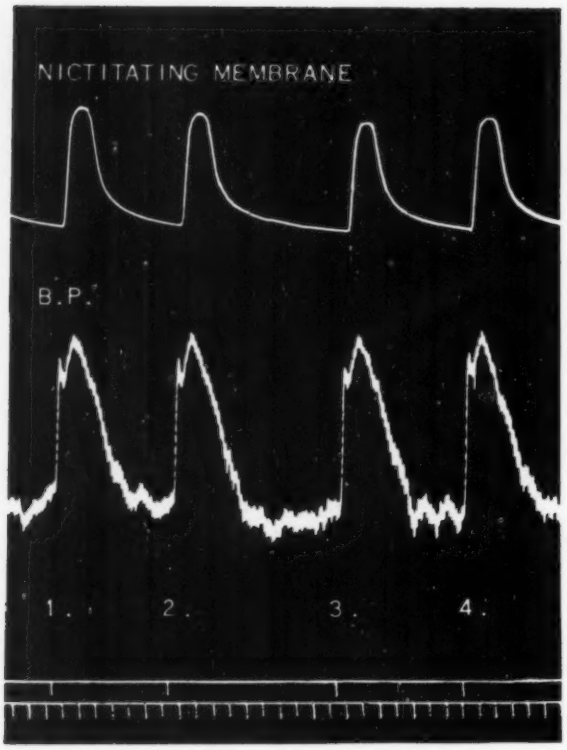


FIG. 5.—Cat anaesthetized with chloralose and pre-treated with atropine, cocaine, and mepyramine. Responses of nictitating membrane (upper record), and of blood pressure (lower record), to i.v. injections of (1) a mixture of 2·5 μg. adrenaline and 0·5 μg. noradrenaline; (2) 0·05 ml. of an extract of adrenals taken from saline-treated control rats; (3) 0·10 ml. of an extract of adrenals taken from rats treated for 14 days with TM 10 (10 mg./kg. daily); (4) a mixture of 2 μg. adrenaline and 1 μg. noradrenaline. Shows that the catechol amine content of the TM 10-treated rat adrenal extract is considerably less than that of the control. Time, 30 sec.

one-half the amount of pressor amines present in the control extract. The amine-content of the two extracts was then estimated colorimetrically, and, from the results shown in Table I, it can be seen that there is satisfactory agreement between the results afforded by the two methods. The amine content is expressed as μg./g. adrenal gland tissue, and average values for the biological and colorimetric results are shown.

It is interesting to note, from the results given in Table I, that the reduction in adrenaline- and noradrenaline-content of the adrenals, resulting from treatment of the rats with TM 10, affects both amines in the same proportion so that the ratio of adrenaline to noradrenaline in the glands remains practically unchanged at about 3 to 1. Thus it would seem improbable that TM 10 affects, in any way, the processes of methylation within the adrenals.

General Effects of TM 10 on Rats

None of the animals treated with TM 10 died, nor did they exhibit loss of weight or signs of ill-health. There were no pathological signs at autopsy. There was, however, a consistent circumscribed loss of hair immediately over the site of each injection which followed about two days after the injection. The reason for this was not obvious, for the drug was administered aseptically on all occasions; no hair-loss occurred in the saline- or choline phenyl ether-treated rats.

DISCUSSION

Quantitative assay of the pressor amine content of the adrenal glands of rats treated with TM 10 daily for 14 days has shown that the amount of adrenaline and noradrenaline present is only about one-half that found in saline-treated control animals. Histological observations on sections cut from the suprarenals support these results by demonstrating a marked reduction in the intensity of the chromaffin reaction of the medullary cells. Reduction in catechol amines is histologically recognizable as early as the fifth day of treatment, and becomes progressively more marked as treatment continues.

TM 10 may act in one of three different ways, viz.: (1), the drug may interfere with biosynthesis by blocking one or more chemical stages in the elaboration of catechol amines; (2), it may have a stimulating action on some part of the nervous system, resulting in increased discharge of splanchnic nerve impulses; (3), it may act as a direct stimulant of the medullary cells whereby adrenaline, noradrenaline, or their precursors, are liberated into the general circulation and the gland thus exhausted of these substances.

Of the three possibilities, it would seem that the first is better supported by the experimental facts than are either the second or third; for, though TM 10 has weak nicotine-like stimulant actions (Hey and Willey, 1954; Willey, 1957) which might result

TABLE I

EFFECT OF TM 10 ON CATECHOL AMINE-CONTENT OF RAT ADRENALS

Amine	Quantity Estimated (μg./g. Gland Tissue)				% Adrenaline in Total
	Bio-assay	Colorimetric	Average	Total Catechols	
Control Group:					
Adrenaline	808	735	771	1,029	75
Noradrenaline ..	267	250	258		
TM 10-treated Group:					
Adrenaline	402	382	392	536	73
Noradrenaline ..	134	155	144		

in direct stimulation of medullary cells, it is unlikely that these play a significant part in the depletion process, since treatment of rats for two weeks with daily injections of choline phenyl ether bromide—a compound with powerful nicotine-like stimulant actions—does not result in histological evidence of catechol amine depletion. Furthermore, the possibility that TM 10 might exert its effect centrally, causing increased splanchnic nerve activity, is ruled out by the experiments on rats with chromaffin tissue grafted into the anterior chamber of the eye. Such grafts are entirely without nervous connexions (Coupland, unpublished observations); nevertheless, they showed marked histological signs of amine depletion in the TM 10-treated rats, whereas grafts obtained from saline- or insulin-treated animals showed no detectable changes (Coupland, unpublished observations).

It is of special interest to recall the observations of Udenfriend and his co-workers, who were able to estimate (by pre-treating rats with ^{14}C-labelled tyrosine or phenylalanine) the approximate time taken for half a given amount of radioactive adrenaline or noradrenaline to disappear from the store of these catechols in the suprarenals. Their results indicated a half turnover time for adrenaline in the rat suprarenal of about 9 days (Udenfriend, Cooper, Clark, and Baer, 1953) and, in later experiments, a half turnover time for both adrenaline and noradrenaline of about seven days (Udenfriend and Wyngaarden, 1956).

On the basis of Udenfriend's work it could, therefore, be predicted that if the biosynthesis of catechol amines in the rat was blocked completely, it would take from 7 to 9 days for the total suprarenal amines to fall to one-half their normal level. Under the conditions of our experiments, where the animals received only one dose of the drug per 24 hr., it is unlikely that biosynthesis could have been blocked sufficiently to achieve the maximum rate of depletion thus predicted. Nevertheless, though the similarity may be coincidental, the remarkably close agreement between the time relationships as revealed by the experiments of Udenfriend *et al.* (1953), and those of the present work—in which a half-depletion of catechol amines occurred after 14 days' treatment with TM 10—suggests to us that the mode of action of TM 10 is one of interference with biosynthesis, rather than one of liberation of stored amines from the cells.

The effects of TM 10 on the rat suprarenal appear to be fully reversible; histological signs of restoration of catechol amines made their appearance by the sixth day after the cessation of TM 10-treatment, and restoration appeared complete by the 14th day. These observations accord fully with the information yielded by Hökfelt's (1951) studies on the re-formation of adrenal catechol amines following depletion by insulin: he showed that about six days were required for full restoration, thus indicating that the rate of synthesis of catechol amines in the rat adrenal medulla is slow.

References

Brown, B. G., and Hey, P. (1956). *Brit. J. Pharmacol.*, **11**, 58.

Coupland, R. E. (1953). *J. Endocrin.*, **9**, 194.

—— (1955). *Nature, Lond.*, **175**, 211.

Edge, N. D., Mason, D. F. J., and Wyllie, J. H. (1957). *Brit. J. Pharmacol.*, **12**, 312.

Euler, U. S. v., and Hamberg, U. (1949). *Science*, **110**, 561.

Exley, K. A. (1957). *Brit. J. Pharmacol.*, **12**, 297.

Hey, P., and Willey, G. L. (1954). Ibid., **9**, 471.

Hillarp, N. Å., and Hökfelt, B. (1954). *Acta physiol. scand.*, **30**, 55.

Hökfelt, B. (1951). Ibid., **25**, Suppl. 92.

Holland, W. C., and Schümann, H. J. (1956). *Brit. J. Pharmacol.*, **11**, 449.

Udenfriend, S., Cooper, J. R., Clark, C. T., and Baer, J. E. (1953). *Science*, **117**, 663.

—— and Wyngaarden, J. B. (1956). *Biochim. et Biophys. Acta*, **20**, 48.

Willey, G. L. (1957). *Brit. J. Pharmacol.*, **12**, 128.

Brit. J. Pharmacol. (1957), **12**, 312.

SOME ACTIONS OF SUBSTITUTED CHOLINE PHENYL ETHERS, PARTICULARLY OF CHOLINE 2:6-XYLYL ETHER

BY

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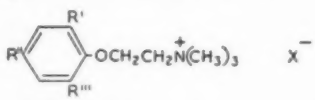
Marked nicotine-like stimulant properties are possessed by choline phenyl ether and choline *o*-tolyl ether, and to a decreasing extent by choline 2:6-xylyl ether and choline 2:4:6-mesityl ether. The compounds all show neuromuscular blocking properties, which are of short duration and pass from mainly decamethonium-like to mainly curare-like as more methyl groups are added to the phenyl nucleus. This series of compounds also possesses muscarinic, weak anti-adrenaline and vasodilator properties, as well as long-lasting local anaesthetic effects in the two compounds tested by intradermal injection.

Hey and Willey (1953, 1954), whilst examining a series of substituted choline phenyl ethers, discovered that some of these, and, in particular, choline 2:6-xylyl ether bromide (TM 10), had local anaesthetic properties, characterized by an unusually long duration of action. Nádor, Herr, Pataky, and Borsy (1953) have also described local anaesthesia of long duration with quaternary ammonium derivatives of well-known local anaesthetics.

Because of the potential clinical usefulness of a compound with this property we have further examined choline 2:6-xylyl ether bromide and compared some of its properties with other closely related quaternary ammonium compounds. As a result of this study and those of Exley (1956), Bain and Fielden (1956), and Willey (1957), other interesting properties in this series have been revealed. Furthermore, choline phenyl ether has a marked and well-known nicotinic effect on the blood pressure (Hunt and Renshaw, 1929; Hey, 1952).

The compounds examined were:



Choline phenyl ether methylsulphate (TM 1) ($R'=R''=R'''=H$; $X=Me_2SO_4$). Choline *o*-tolyl ether bromide (TM 18) ($R'=CH_3$; $R''=R'''=H$; $X=Br$). Choline 2:6-xylyl ether bromide (TM 10) ($R'=R'''=CH_3$; $R''=H$; $X=Br$). Choline 2:4:6-mesityl ether bromide (TM 17) ($R'=R''=R'''=CH_3$; $X=Br$).

The unsubstituted compound has long been known as choline phenyl ether, and Hey and Willey (1953, 1954) used a similar nomenclature for TM 10. To avoid confusion we have adopted these names and used the same system in naming the other members of the series. The third and fourth compounds listed above were first obtained from Dr. P. Hey. Later supplies, as well as the other two compounds listed, were kindly prepared by our colleague, Dr. K. Gaimster.

METHODS

Acute toxicities were determined in groups of 10 albino mice. Mice of either sex weighing 16 to 24 g. were used. LD50, limits of errors, and the slopes of the regression lines were determined graphically.

Local anaesthesia was tested by three methods. These were the guinea-pig weal and frog lumbar plexus methods of Bülbring and Wajda (1945), and the guinea-pig corneal method. In the first of these, observations on groups of four or six guinea-pigs were performed every 5 min. for 30 min. and subsequently at 10 or 15 min. intervals until anaesthesia became minimal. Activities were determined by comparison of the response/time graphs plotted for each experiment, cocaine and sometimes lignocaine being used as standards for comparison.

Experiments on the blood pressure and neuromuscular transmission were performed in cats under chloralose anaesthesia or in spinal preparations; the compounds were injected intravenously. In experiments on the superior cervical ganglion-nictitating

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membrane preparation intra-arterial injections were employed. The cervical sympathetic nerve trunk was stimulated peripherally at 20/sec. and the sciatic nerve at 6/min. or 50/sec. using supramaximal rectangular pulses of 0.5 msec. duration.

Anti-adrenaline activity was examined using the perfused rabbit ear preparation in the manner described by Fleckenstein (1952). The concentration of drug was found which reduced the sensitivity of the preparation to adrenaline by a factor of 10. The perfusing fluid was that recommended by Page and Green (1948).

Other methods are described with the results. Cocaine, lignocaine, and adrenaline were used as the hydrochlorides, acetylcholine and (+)-tubocurarine as the chlorides, hexamethonium as the bromide, gallamine as the triethiodide, neostigmine as the methyl sulphate, atropine as the sulphate, histamine as the acid phosphate, and nicotine as the hydrogen tartrate. All doses in the text refer to these salts unless otherwise stated.

Results

Toxicity in Mice

The acute intravenous toxicities are shown in Table I. The acute subcutaneous LD50 of choline 2:6-xylyl ether was 240 mg./kg. compared with 8.5 mg./kg. intravenously. The toxic symptoms included muscular weakness and clonic convulsions. In addition, choline phenyl ether produced muscular twitching. After intravenous injection death occurred within a few minutes and followed respiratory arrest.

TABLE I

ACUTE INTRAVENOUS TOXICITY OF SUBSTITUTED CHOLINE PHENYL ETHERS IN MICE

Compound	LD50 (mg./kg.)	% Limits of Error ($P=0{\cdot}05$)	Slope of Regression Line
Choline phenyl ether methylsulphate	2·20	82·0–121·5	4·8
Choline *o*-tolyl ether bromide	0·87	90·5–110·5	14
Choline 2:6-xylyl ether bromide	8·50	90·5–110·5	12·4
Choline 2:4:6-mesityl ether bromide	7·80	90·5–111·0	12·5

Local Tolerance

Injected intradermally into the shaved backs of guinea-pigs and into the ears of rabbits, 0.2 ml. of a 2.0% w/v solution of choline 2:6-xylyl ether caused a slight erythema, which resolved in two or three days. In one rabbit, a few small petechiae were found after close examination with a lens.

Local Anaesthetic Effects

Guinea-pig Weal.—We have confirmed the finding of Hey and Willey (1954) that choline 2:6-xylyl ether produces long-lasting local anaesthesia. In our experiments, the compound was compared directly with both cocaine and lignocaine. The results obtained with the latter two compounds were practically identical when the same concentrations were used.

Using 0.33% solutions, both cocaine and lignocaine produced virtually complete anaesthesia within 5 min., which persisted for 30 min. and then declined fairly rapidly. Choline 2:6-xylyl ether did not produce complete anaesthesia until 20 to 30 min. after injection and this was followed by a slow return of sensation (Fig. 1*a*). In this

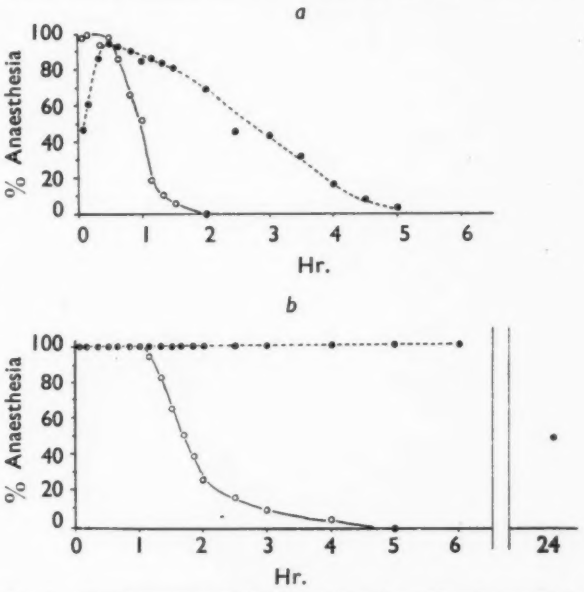


FIG. 1.—Time/response relationship of intradermal local anaesthesia produced in guinea-pigs by cocaine and choline 2:6-xylyl ether. (*a*), Mean results on 12 guinea-pigs (2 experiments) using 0.33% solutions. (*b*), Mean results on 4 guinea-pigs using 3.0% solutions. ○——○, cocaine; ●- - -● choline 2:6-xylyl ether. Ordinates, % anaesthesia. Abscissae, time in hr.

detail, our results differed from those of Hey and Willey (1954), who found that this concentration produced a 75% effect within 5 min. of injection.

When the concentration was increased to 1.0%, we found no appreciable modification of the response to cocaine, but the response to choline 2:6-xylyl ether was much quicker in onset and the duration of anaesthesia was also increased. A concentration of 3.0% produced some increase in the duration of anaesthesia with cocaine, whereas choline 2:6-xylyl ether produced complete anaesthesia for more than 6 hr. and sensation was still reduced after 24 hr. (Fig. 1*b*).

We have also examined choline 2:4:6-mesityl ether by this method. Again it was found that, with a concentration of 0.33%, maximal anaesthesia occurred only after an interval of 30 to 40

min. With this concentration there was complete recovery only after 4½ hr.

Guinea-pig Cornea.—By this method choline 2:6-xylyl ether had no activity when a 2.0% solution was continuously applied to the cornea for 10 min. This agrees with the results of Hey and Willey (1954) in the rabbit.

Frog Lumbar Plexus.—On this preparation, a 0.8% solution of choline 2:6-xylyl ether was effective only after 23 min. contact, whereas a 0.1% solution of cocaine produced anaesthesia in not more than 14 min.

Cardiovascular Effects

Effects on Blood Pressure and Heart Rate.—In the cat under chloralose anaesthesia, the rise in pressure produced by 0.1 mg./kg. of choline phenyl ether was preceded by a brief fall. The rise was abolished by hexamethonium (10 mg./kg.), leaving only a small depressor response which could be inhibited by atropine (3 mg./kg.) (Fig. 3).

Choline *o*-tolyl ether also produced profound effects with doses of 0.05 mg./kg. and greater. These consisted in a brief period of bradycardia followed by tachycardia and a rise of blood pressure. For example, a dose of 0.3 mg./kg. produced a fall in heart rate from 96/min. to 60/min. followed by a rise to 144/min. accompanied by a rise in blood pressure of 90 mm. Hg. This response was regularly obtained (Fig. 2). The pressor response was greatly reduced (to less than 10 mm. Hg) although not abolished by hexamethonium (10 mg./kg.). After hexamethonium, the compound did not cause a fall in blood pressure.

Choline 2:6-xylyl ether had a complex action (Fig. 3). Initially, provided a moderately large dose of 2 mg./kg. or more was given, there was a very brief fall in pressure followed by a marked rise which lasted for 4 to 5 min. The heart rate fell during the latter part of the response; in one experiment an initial heart rate of 200/min. was reduced to 168/min. Subsequent doses caused only a marked fall in blood pressure of 30 to 60 sec. duration. If an initial dose caused only a fall in blood pressure, then a pressor response could not subsequently be obtained. Despite tachyphylaxis of the pressor action seen with this com-

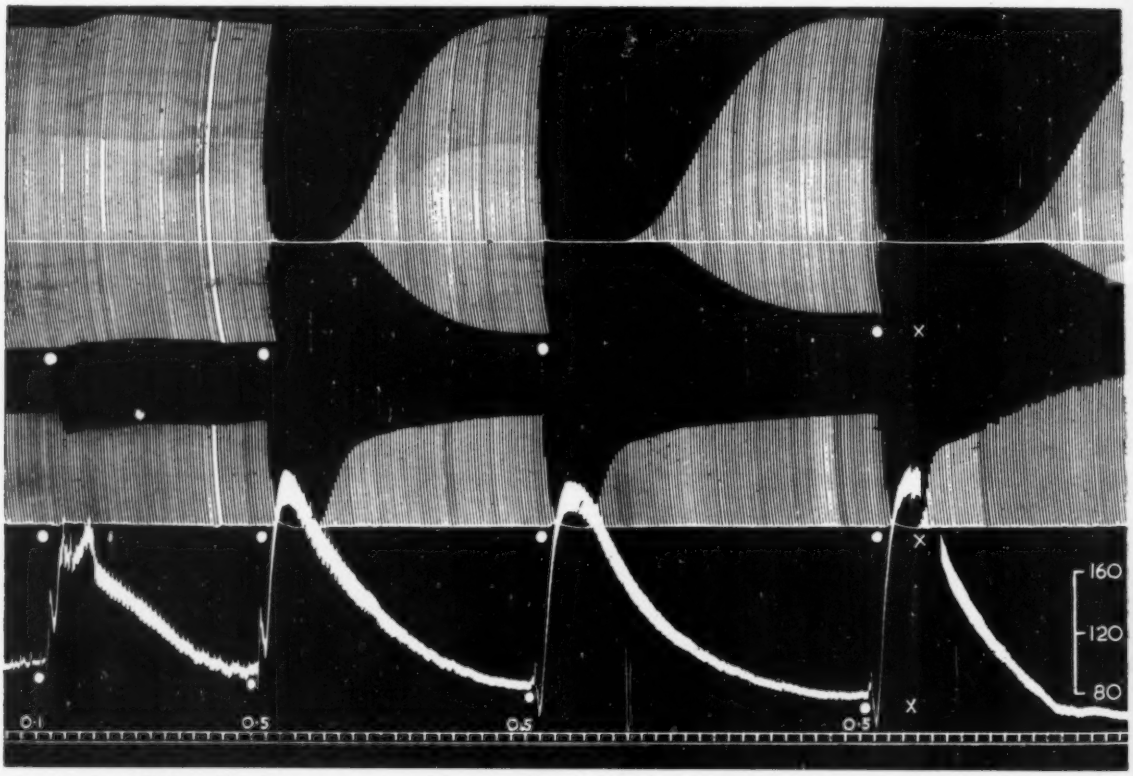


FIG. 2.—Cat, 3.5 kg., chloralose anaesthesia. Effect of choline *o*-tolyl ether on neuromuscular transmission and blood pressure. Tracings from above downwards: contractions of tibialis; of soleus; blood pressure; time marker. At ●, i.v. injections of choline *o*-tolyl ether in doses shown in mg./kg. Each injection was followed by micturition. At X, 0.1 mg./kg. neostigmine. Vertical scale, blood pressure in mm. Hg. Time, 30 sec.

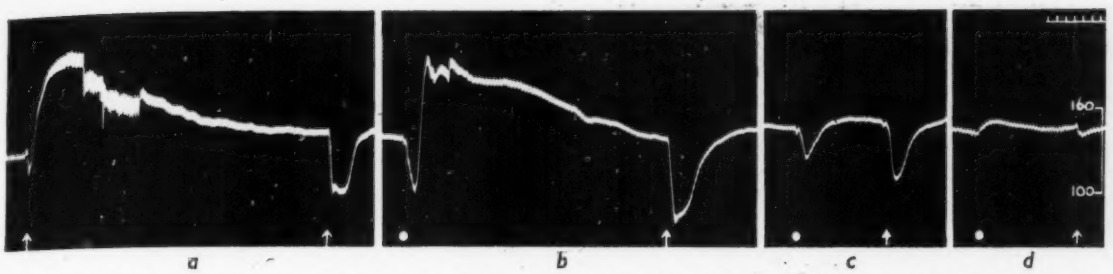


FIG. 3.—Cat, 2.5 kg., chloralose anaesthesia. Blood pressure record. At arrows, 2 mg./kg. choline 2:6-xylyl ether. At ●, 0.1 mg./kg. choline phenyl ether. (*a*), Note that only the first dose of choline 2:6-xylyl ether caused a pressor response. (*b*), A pressor response was still obtained with choline phenyl ether. Between (*b*) and (*c*) 10 mg./kg. of hexamethonium was given. Between (*c*) and (*d*) 3 mg./kg. of atropine was administered. Vertical scale, blood pressure in mm. Hg. Time, 10 sec.

pound, a nicotine-like pressor response could still be obtained with choline phenyl ether (Fig. 3). The depressor response obtained with choline 2:6-xylyl ether could be inhibited by atropine. (This muscarinic property was not reported by Hey and Willey (1954), because they worked on spinal cats which had received atropine). The effect of peripheral stimulation of the vagus nerve on the blood pressure and heart rate was temporarily reduced when doses of about 8 mg./kg. were employed.

Choline 2:4:6-mesityl ether had very little pressor action in the cat under chloralose anaesthesia. Usually only a fall in blood pressure was observed with doses of 1 to 10 mg./kg. This fall was sometimes preceded by a slight rise in pressure. The depressor response could not be abolished by atropine although some reduction of the response occurred. In the spinal cat, doses of 2 to 10 mg./kg. caused only a rise in blood pressure. No tachyphylaxis of this pressor action was observed.

Effects on the Perfused Rabbit Ear.—Choline 2:6-xylyl ether has some anti-adrenaline activity on the cat blood pressure (Hey and Willey, 1954). This property is dealt with in some detail by Exley (1956) and Bain and Fielden (1956). In our experiments on the isolated perfused rabbit ear, the compounds caused vasodilatation and exhibited weak anti-adrenaline activity (Fig. 4). There was little difference between the potency of the four compounds, the concentrations required to reduce the constrictor action of adrenaline to 1/10 ranging from 50 to 150 μg./ml. Choline phenyl ether was the least active and choline *o*-tolyl ether the most active compound.

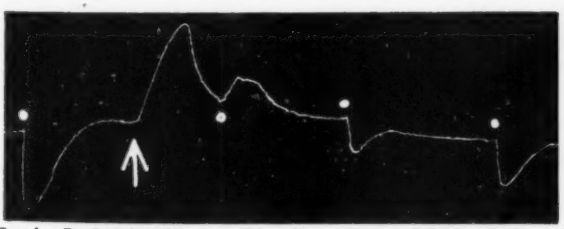


FIG. 4.—Perfused rabbit ear. The effect of 1 mg. of choline 2:6-xylyl ether, given at arrow, which caused vasodilatation, on responses to 0.05 μg. adrenaline (●).

Effects on Superior Cervical Ganglion

It was observed in the cat that choline 2:6-xylyl ether and choline *o*-tolyl ether caused micturition and a brief but copious flow of saliva, even when the pressor response to the former compound could no longer be obtained. We considered that these effects were probably due to nicotine-like stimulation and decided to examine the compounds on the superior cervical ganglion-nictitating membrane preparation of the cat under chloralose anaesthesia, using intra-arterial injections.

Stimulant effects were repeatedly obtained with choline phenyl ether and choline *o*-tolyl ether in doses as low as 10 μg. Large doses (100 to 400 μg.) of these two compounds caused a biphasic contraction of the nictitating membrane (Fig. 5*c*), the secondary contraction occurring about 20 to 25 sec. after the primary spike contraction. This secondary contraction was probably due to the release of adrenaline from the adrenal medulla, but may have been a muscarinic action directly on the membrane. It could be eliminated if the blood supply to the membrane was interrupted by clamping the external carotid artery during and after the injection.

The results obtained with choline 2:6-xylyl ether and choline 2:4:6-mesityl ether were very variable. Sometimes no stimulation could be obtained with doses ranging from 10 to 400 μg. In preparations where stimulation was obtained, sometimes with doses as small as 20 μg., sensitivity rapidly declined until no effect could be obtained with doses up to 400 μg. Similarly, in preparations where the preganglionic fibres were stimulated electrically, choline 2:6-xylyl ether produced a brief relaxation of the membrane, but

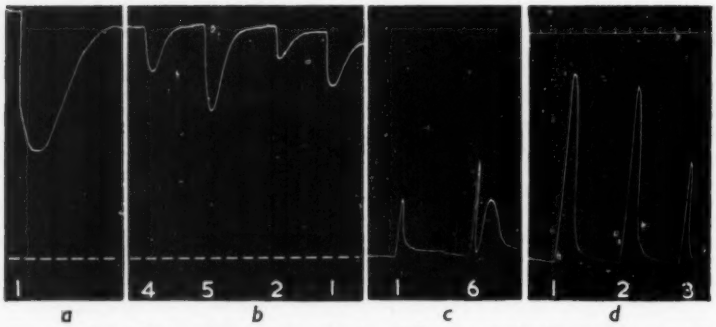


FIG. 5.—Cat, 3.0 kg., chloralose anaesthesia. Effect on nictitating membrane. All injections were given intra-arterially to superior cervical ganglion. (*a*) and (*b*), continuous preganglionic stimulation of cervical sympathetic nerve trunk; (*c*) and (*d*), no electrical stimulation. At (1), 200 μg., at (2), 100 μg., at (3), 50 μg. choline 2:6-xylyl ether. At (4), 4 μg., at (5), 8 μg. hexamethonium. At (6), 200 μg. choline phenyl ether. Note decreased sensitivity to choline 2:6-xylyl ether between (*a*) and (*b*) and potentiation of stimulant effect of this compound by choline phenyl ether between (*c*) and (*d*). Time, 60 sec.

the response to repeated injections rapidly declined. Both effects are shown in Fig. 5, where 200 μg. of choline 2:6-xylyl ether at first produced marked relaxation of the membrane stimulated preganglionically. Sensitivity to the compound rapidly declined, but, when preganglionic stimulation was withdrawn, injection of the same dose caused the membrane to contract. Since both the relaxation and the contraction were rapid in onset, and appeared even though the clamp on the external carotid artery was left in position, they are probably due to a direct effect on the ganglion. Hey and Willey (1954) have shown that this compound has no stimulating action on the nictitating membrane after removal of the superior cervical ganglion, but they were again using animals treated with atropine, so that muscarinic effects would not be seen. In one experiment, the stimulating action of choline 2:6-xylyl ether was potentiated by choline phenyl ether (Fig. 5*c* and *d*).

Effects on Isolated Guinea-pig Ileum

On the isolated guinea-pig ileum suspended in a 5 ml. bath containing oxygenated Tyrode solution, choline phenyl ether and choline *o*-tolyl ether produced submaximal contractions in concentrations of the order of 2 μg./ml. The other two compounds produced comparable contractions in concentrations of the order of 10 to 20 μg./ml.

Contractions following choline phenyl ether and nicotine were blocked by hexamethonium (100 μg./ml.), leaving contractions elicited by acetylcholine and histamine unaffected.

In other experiments, the effect of choline *o*-tolyl ether was reduced or abolished by hexamethonium. When only reduction of the response was obtained, the effect of nicotine was reduced to a greater extent. Atropine (1.0 μg./ml.) greatly reduced the effects of approximately equipotent doses of choline *o*-tolyl ether and nicotine.

It was not possible to block the effects of choline 2:6-xylyl ether or choline 2:4:6-mesityl ether with hexamethonium, although the effects of both these compounds and of nicotine were reduced or abolished by atropine (Fig. 6).

Neuromuscular Blocking Effects

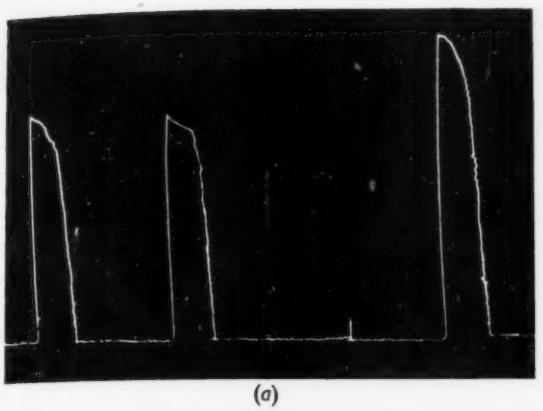
One of the effects noted in mice after intravenous injection of choline 2:6-xylyl ether was muscular weakness and slowed respiration. In the cat under chloralose anaesthesia, doses greater than about 3 mg./kg. caused a brief apnoea. It was therefore decided to examine these compounds for neuromuscular blocking properties.

Effects on Soleus and Tibialis Muscles of the Cat.—The neuromuscular blocking effect seen in the cat under chloralose anaesthesia was one of the most interesting properties of these compounds. With all four compounds the block rapidly reached a maximum and was of short duration.

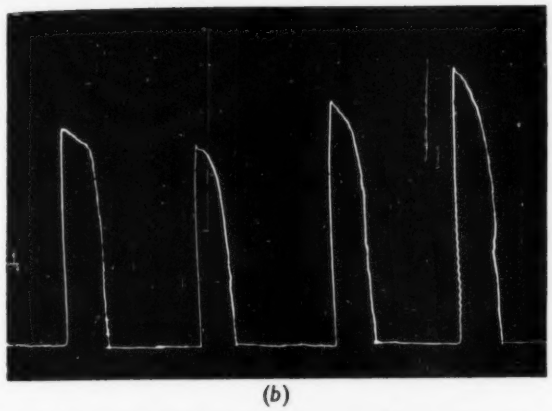
In a dose of 5.0 mg./kg., choline phenyl ether abolished the contractions of both the tibialis and soleus muscles in response to nerve stimulation.

Choline *o*-tolyl ether can block neuromuscular transmission (Exley, 1954). In one preparation, doses of 0.05 and 0.10 mg./kg. caused a brief potentiation followed by a slight reduction of the twitch of soleus and potentiated the twitch of tibialis. The contractions of both muscles were completely inhibited by 0.5 mg./kg. This block lasted for 2.5 min. and recovery was rapid (Fig. 2). Injection of 0.1 mg./kg. neostigmine had little or no effect on the rate of recovery with this compound and in one experiment the relative sensitivity of the two muscles was reversed.

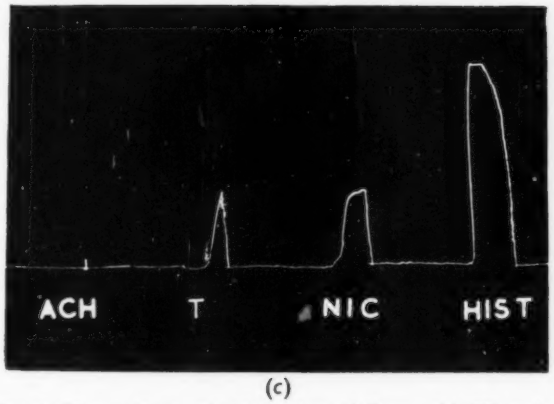
Neuromuscular block appeared with doses of 3 to 5 mg./kg. of choline 2:6-xylyl ether. In the majority of experiments the contractions of soleus were reduced less than were those of tibialis, although the relative sensitivity of the two muscles was sometimes reversed. In no experiment was an initial stimulation of either muscle observed. Fig. 7 shows an experiment in which 7.0 mg./kg. of choline 2:6-xylyl ether



(*a*)



(*b*)



(*c*)

FIG. 6.—Contractions of isolated guinea-pig ileum. (*a*), 100 μg./ml. hexamethonium present throughout; (*b*), Tyrode solution only; (*c*), 1.0 μg./ml. atropine present throughout. ACh, 0.1 μg. acetylcholine; T, 100 μg. choline 2:6-xylyl ether; NIC, 100 μg. nicotine; HIST, 0.05 μg. histamine. The stimulant drugs were in contact with the ileum for 30 sec. Bath volume, 5 ml.

produced an almost complete inhibition of the contractions of tibialis. The contractions had increased to 50% of the pre-injection height within 6 min. and fully recovered within about 15 min. By comparison, gallamine in a dose of 1.2 mg./kg. produced an almost complete neuro-

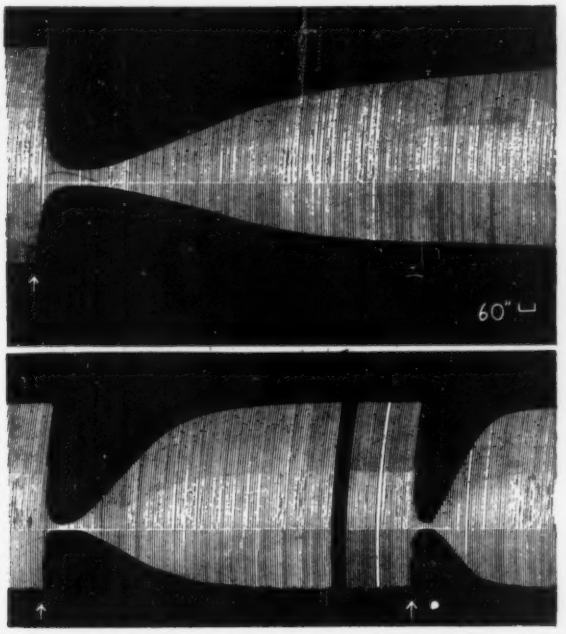


FIG. 7.—Cat, 2.70 kg., chloralose anaesthesia. Effect of 2:6-xylyl ether and gallamine given intravenously on contractions of tibialis muscle in response to stimulation of the sciatic nerve. Top: 1.2 mg./kg. gallamine at arrow. Bottom: 7.0 mg./kg. choline 2:6-xylyl ether at arrows. At ●, 0.15 mg./kg. neostigmine. Note short duration of effect produced by choline 2:6-xylyl ether and increased speed of recovery after injection of neostigmine. Time, 60 sec.

muscular block with 50% recovery in 12 min.; full recovery was not reached after 35 min. Several experiments have shown that choline 2:6-xylyl ether has approximately 0.25 of the potency and duration of action of gallamine. During block due to choline 2:6-xylyl ether, a tetanus was poorly maintained and was followed by partial reversal of the block; neostigmine hastened recovery.

Choline 2:4:6-mesityl ether was much less potent in neuromuscular blocking properties. A dose of 10 mg./kg. was required to produce 20% block of both muscles. 20 mg./kg. produced complete block of short duration, reversible by a tetanus.

Effects on Frog Rectus Abdominis Muscle.—Choline phenyl ether and choline *o*-tolyl ether both caused contracture of the isolated frog rectus abdominis muscle suspended in oxygenated frog Ringer solution. Their activities were about 10% and 35% respectively of the activity of acetylcholine and the contractures were blocked by (+)-tubocurarine.

Choline 2:6-xylyl ether had mixed actions. Concentrations up to 0.2 mg./ml. present in the

bath for 1 min. reduced or abolished the subsequent effect of acetylcholine. Higher concentrations (0.2 to 1 mg./ml.) caused a contracture.

Choline 2:4:6-mesityl ether blocked acetylcholine in concentrations up to 1 mg./ml., but showed no stimulant effect.

Anti-cholinesterase Activity.—Choline phenyl ether, choline 2:6-xylyl ether, and choline 2:4:6-mesityl ether had little effect upon the activity of red cell or plasma cholinesterases derived from defibrinated horse blood, and a concentration of approximately 10^{-2}M was required to produce 50% inhibition using a manometric method.

Discussion

Our results suggest that nicotinic properties occur in this series of compounds. Thus choline phenyl ether and choline *o*-tolyl ether caused a pressor response which could be blocked by hexamethonium. When they were injected intra-arterially to the superior cervical ganglion, all four compounds produced a contraction of the nictitating membrane, even though the external carotid artery was clamped, so that a peripheral action was unlikely. However, results with choline 2:6-xylyl ether and choline 2:4:6-mesityl ether were variable, especially on the superior cervical ganglion. On the isolated guinea-pig ileum, choline phenyl ether and choline *o*-tolyl ether produced a contraction which could be abolished or reduced by hexamethonium. On the isolated frog rectus-abdominis muscle choline 2:4:6-mesityl ether was the only drug which did not produce a contracture.

These nicotinic properties were most pronounced with the unsubstituted compound and decreased with increasing substitution on the ring. It is not clear from the present work why tachyphylaxis of the nicotine-like properties of choline 2:6-xylyl ether, seen both on the blood pressure and on the superior cervical ganglion, should leave responses to choline phenyl ether unaffected. Similar observations have been reported by Willey (1957), who showed that nicotine gave a pressor response when choline 2:6-xylyl ether no longer did so.

Muscarinic properties were also seen in this series. Thus choline phenyl ether and choline 2:6-xylyl ether produced a fall in blood pressure after the nicotinic effects had been eliminated either by hexamethonium or through tachyphylaxis respectively. The depressor response could then be abolished by atropine. Furthermore, on the guinea-pig ileum, choline 2:6-xylyl ether and choline 2:4:6-mesityl ether both produced a contraction which was unaffected by hexamethonium but was abolished or reduced by atropine. Since the contractions produced by the other two compounds were blocked by hexamethonium it was not possible to carry out an unequivocal test for muscarinic effects on this preparation.

The compounds also showed neuromuscular blocking properties. Bovet, Depierre, and Lestrange (1947) and Winter and Lehman (1950) have previously reported this property with choline phenyl ether. The former authors found that 2 to 5 mg. injected intravenously in a rabbit which was receiving artificial respiration caused neuromuscular block which lasted for 20 min. Although we have not examined this compound quantitatively for its neuromuscular blocking action in the cat, evidence from the intravenous toxicity in mice, the relative potency on the frog rectus abdominis muscle, and the doses employed by Bovet *et al.* (1947) to produce neuromuscular paralysis in the rabbit, suggest that this compound is less active than choline *o*-tolyl ether. Both of these compounds were short-acting and predominantly decamethonium-like in action. Choline 2:6-xylyl ether was less active still and its actions were predominantly like those of tubocurarine. This change in mode of action was still allied to a short duration of effect. We have not completely analysed the mode of action of this compound and it is conceivable that neuromuscular paralysis might be due to the prevention of acetylcholine release from the motor nerve endings. However, this possibility seems unlikely in view of the long duration of its local anaesthetic action. A short neuromuscular block was also seen with choline 2:4:6-mesityl ether which had a weak, mainly tubocurarine-like, type of action. This compound also possesses prolonged local anaesthetic properties.

Brown and Hey (1956) have demonstrated inhibition of amine oxidase by compounds in this series; choline phenyl ether was the least active, whereas choline *o*-tolyl ether and choline 2:4:6-mesityl ether possessed high activity and were more active than choline 2:6-xylyl ether. In addition these compounds possess weak anti-adrenaline properties, first described by Hey and Willey (1954).

We are indebted to our colleagues, Dr. H. W. Reading and Mrs. M. Davies, for the anticholinesterase determinations.

References

Bain, W. A., and Fielden, R. (1956). *J. Physiol.*, **133**, 70P.

Bovet, D., Depierre, F., and de Lestrange, Y. (1947). *C.R. Acad. Sci., Paris*, **225**, 74.

Brown, B. G., and Hey, P. (1956). *Brit. J. Pharmacol.*, **11**, 58.

Bülbring, E., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 78.

Exley, K. A. (1954). Cited by Hey, P., and Willey, G. L. (1954). *Brit. J. Pharmacol.*, **9**, 471.

—— (1956). *J. Physiol.*, **133**, 70P.

Fleckenstein, A. (1952). *Brit. J. Pharmacol.*, **7**, 553.

Hey, P. (1952). Ibid., **7**, 117.

—— and Willey, G. L. (1953). *J. Physiol.*, **122**, 75P.

—— —— (1954). *Brit. J. Pharmacol.*, **9**, 471.

Hunt, R., and Renshaw, R. R. (1929). *J. Pharmacol.*, **35**, 99.

Nádor, K., Herr, F., Pataky, Gy., and Borsy, J. (1953). *Nature, Lond.*, **171**, 788.

Page, I. H., and Green, A. A. (1948). *Meth. med. Res.*, **1**, 123.

Willey, G. L. (1957). *Brit. J. Pharmacol.*, **12**, 128.

Winter, C. A., and Lehman, J. T. (1950). *J. Pharmacol.*, **100**, 489.

Brit. J. Pharmacol. (1957), **12**, 320.

THE ENZYMATIC HYDROLYSIS OF IMIDAZOLEACRYLOYLCHOLINE (MUREXINE) AND IMIDAZOLEPROPIONYLCHOLINE (DIHYDROMUREXINE) BY VARIOUS CHOLINESTERASES

BY

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Several choline esters, including the imidazoleacryloyl and imidazolepropionyl compounds, have been hydrolysed by cholinesterases from various sources. The imidazolepropionyl ester was metabolized by cholinesterases obtained from human plasma, ox spleen, ox serum, and guinea-pig liver, but not by rat liver or bovine red cell cholinesterase. It is suggested the imidazolepropionylcholine or a closely related ester might be the natural substrate for "non-specific" cholinesterase.

Vincent and Jullien (1938) reported that extracts of the hypobranchial gland of *Murex* caused contraction of the leech muscle. This action was abolished by incubating these extracts in serum, an indication that the substance which caused the contraction was metabolized. Later, Erspamer and Dordoni (1947) described the presence of a choline ester in the hypobranchial gland of the snail, *Murex trunculus*. This ester was identified (Erspamer and Benati, 1953) as (2-β-imidazol-4(5)-ylacryloyloxyethyl)trimethylammonium chloride hydrochloride variously named murexine, urocanylcholine, or imidazoleacryloylcholine (ImAcrCh). ImAcrCh and its analogue, imidazolepropionylcholine (ImPrCh), stimulate autonomic ganglia, interfere with transmission at the neuromuscular junction, and potentiate the effects of histamine on smooth muscle (Erspamer and Dordoni, 1947; Kewitz, 1955; Conroy, Kappell, Ferruggia, and Randall, 1956; Tabachnick and Roth, 1957; Winbury, Wolf, and Tabachnick, 1957).

Since Grunert and Kewitz (1955) have demonstrated that an imidazoleacylcholine ester occurs naturally in horse, rat, and bovine brain, the study of the hydrolysis of imidazoleacylcholine esters assumes some significance. Therefore, it was of interest to ascertain whether ImAcrCh and its analogue, ImPrCh, would serve as substrates for several cholinesterases.

MATERIALS AND METHODS

The esterase determinations were performed by a modification of the method of Ammon (1933) at 37° with a gas phase of 95% nitrogen and 5% carbon dioxide. Sufficient $NaHCO_3$ was used to give a final concentration of 0.07M, *p*H 8.0.

Enzyme, buffer and water were placed in the main compartment and substrate and inhibitor in the sidearms. After gassing and temperature equilibration, the substrate was added. If an inhibitor was present, it was added from the sidearm simultaneously with the substrate. In most experiments, readings were taken at 3 to 6 min. intervals.

Various cholinesterases were used. Acetylcholinesterase was prepared from bovine red blood cells (Winthrop-Stearn). Cholinesterase of human plasma (Cholase^R) was obtained as a concentrate, 1 ml. of the preparation representing the cholinesterase content of 350 ml. of original plasma. Rat and guinea-pig liver esterases were prepared by homogenizing 1 part minced liver in 4 parts of cold 0.85% NaCl in a Potter–Elvejhem homogenizer. Bovine spleen esterase was obtained by removing the outer sheath and fat from a portion of spleen and preparing the underlying tissue as described for rat and guinea-pig liver. Bovine serum and spleen were obtained from freshly killed steers.

The following substrates were used: Imidazoleacryloylcholine chloride hydrochloride; imidazolepropionylcholine bromide (Hoffmann-LaRoche); butyrylcholine chloride (J. H. DeLamar and Son); propionylcholine iodide (Dajac Laboratories);

benzoylcholine chloride (J. H. DeLamar and Son); triacetin (Eastman); and methacholine (Sharpe and Dohme).

Results

The velocities of hydrolysis of certain choline esters in final concentrations of 10^{-2}M to $10^{-3.5}$M are given in Table I. ImPrCh and butyrylcholine (BuCh) were hydrolysed at approximately equal rates by human plasma non-specific cholinesterase. The respective Km values for these esters calculated from initial velocities were 1.37×10^{-3}M and 1.53×10^{-3}M. Other substrates tested in this system in decreasing order of activity were propionylcholine (PrCh), benzoylcholine (BzCh), and ImAcrCh. Methacholine (MeCh) was hydrolysed by specific cholinesterase while ImAcrCh and ImPrCh were not metabolized by this enzyme. The inhibition of the hydrolysis of ImPrCh and BuCh by 10^{-5}M eserine with a preparation of human cholinesterase was 87 and 81% respectively.

TABLE I

THE VELOCITIES OF THE ENZYMATIC HYDROLYSIS OF SOME CHOLINE ESTERS BY BOVINE RED BLOOD CELL (SPECIFIC) CHOLINESTERASE (15 UNITS/FLASK) OR BY HUMAN PLASMA (NON-SPECIFIC) CHOLINESTERASE (0.2 ML. OF A 1:375 DILUTION OF CHOLASE/FLASK)

Final volume 2 ml.

Substrate	Cholinesterase	Initial Velocities Expressed as μl.CO_2 Evolved/1 hr.			
		10^{-2}M	$10^{-2.5}$M	10^{-3}M	$10^{-3.5}$M
Methacholine	Specific	235	186	118	36
Imidazolepropionylcholine	,,	—	0	0	0
Imidazoleacryloylcholine	,,	0	0	0	0
Imidazolepropionylcholine	Non-specific	980	735	305	150
Imidazoleacryloylcholine	,,	40	55	61	37
Benzoylcholine	,,	280	295	140	48
Propionylcholine	,,	737	566	300	54
Butyrylcholine	,,	990	775	375	—

Various tissues were examined for their cholinesterase activity (Table II). Guinea-pig liver hydrolysed both BzCh and ImPrCh. Rat liver did not hydrolyse ImPrCh but metabolized triacetin at a very high rate. Some activity was observed with BuCh. Ox serum- and spleen-cholinesterases hydrolysed ImPrCh and BuCh, but not BzCh nor ImAcrCh. The rate of hydrolysis of ImPrCh was somewhat greater than that of BuCh.

TABLE II

THE ENZYMATIC HYDROLYSIS OF CERTAIN CHOLINE ESTERS BY VARIOUS TISSUES

Enzyme/flask: 1 ml. of 20% homogenate of ox spleen, rat or guinea-pig liver, or 1 ml. of ox serum. Final volume 2 ml. (*a*) denotes that the substrate concentration was $10^{-2.5}$M, (*b*) that 15 min. readings and (*c*) that 3 min. readings were extrapolated to obtain the 30 min. values.

Substrate (10^{-2}M)	Eserine (10^{-5}M)	μl. CO_2 Evolved/30 Min./Flask			
		Ox Spleen	Ox Serum	Rat Liver	G. Pig Liver (*b*)
Imidazoleacryloylcholine	0	0	0		
Imidazolepropionylcholine	0	35	36	0	152
,,	+				0
Butyrylcholine	0	25	27	45	
Benzoylcholine	0	0	0		176 (*a*)
,,	+				164 (*a*)
Methacholine	0	24			
Triacetin	0			1,960(*c*)	

A concentration of 10^{-5}M eserine, which inhibited the hydrolysis of BzCh by guinea-pig liver esterase only slightly, completely inhibited the hydrolysis of ImPrCh (Table II).

Discussion

The work reported here suggests some specificity of the "non-specific" cholinesterase for ImPrCh. The evidence for this is as follows: (*a*) The non-specific cholinesterase of human serum hydrolyses ImPrCh more rapidly than ImAcrCh, PrCh, and BzCh. However, BuCh is metabolized slightly more rapidly than ImPrCh. (*b*) ImPrCh and ImAcrCh are not metabolized by the specific esterase of bovine red blood cells, whereas BuCh and BzCh are metabolized by specific cholinesterase though at a slow rate (Adams, 1949). (*c*) The non-specific cholinesterase of ruminant serum and spleen which does not act on BzCh (Gunter, 1946), but which does metabolize BuCh (Bannister, Whittaker, and Wijesundera, 1953; Mendel and Myers, 1955; Hardwick, 1956), has now been shown to hydrolyse ImPrCh more rapidly than BuCh. (*d*) Rat liver homogenates were shown to metabolize triacetin rapidly, BuCh very slowly, and ImPrCh not at all. (*e*) Sawyer (1945) reported the presence of a benzoylcholinesterase in guinea-pig liver which was not inhibited by eserine. We have confirmed this, but have observed also that this tissue rapidly metabolized ImPrCh. The hydrolysis of ImPrCh was prevented by eserine (10^{-5}M final concentration), and therefore the enzyme which metabolized ImPrCh cannot be benzoylcholinesterase.

It would appear that ImPrCh might be as effective as BuCh as a substrate for non-specific cholinesterase. It is conceivable that the specificity pattern displayed by ImPrCh for some cholinesterases would also be displayed by an imidazoleacylcholine ester that occurs in the organism. If this were the case, one might infer a need for the so-called non-specific esterase, namely, for the metabolism of an imidazoleacylcholine ester.

Since this paper was written Foldes, Erdös, Baart, and Shanor (1957) have reported the hydrolysis of imidazoleacryloylcholine and imidazolepropionylcholine by human plasma and red cell cholinesterase.

The authors are grateful to Dr. L. Randall and Dr. V. Erspamer for their generous gifts of imidazolepropionylcholine bromide and imidazoleacryloylcholine chloride hydrochloride respectively, and to Dr. V. Seeberg, Cutter Laboratories, for his kindness in making the Cholase[R] available. The authors wish to express their appreciation to Miss J. Mershon for her assistance.

References

Adams, D. H. (1949). *Biochem. biophys. acta*, **3**, 1.
Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.*, **233**, 486.
Bannister, J., Whittaker, V. P., and Wijesundera, S. (1953). *J. Physiol.*, **121**, 55.
Conroy, C., Kappell, B., Ferruggia, T., and Randall, L. O. (1956). *Fed. Proc.*, **15**, 412.
Erspamer, V., and Benati, O. (1953). *Science*, **117**, 161.
Erspamer, V., and Dordoni, F. (1947). *Arch. int. Pharmacodyn.*, **74**, 263.
Foldes, F. F., Erdös, E. G., Baart, N., and Shanor, S. P. (1957). *Proc. Soc. exp. Biol., N.Y.*, **94**, 500.
Grunert, G., and Kewitz, H. (1955). *Naturwissenschaften*, **42**, 628.
Gunter, J. M. (1946). *Nature, Lond.*, **157**, 359.
Hardwick, D. C. (1956). *Biochem. J.*, **64**, 1P.
Kewitz, H. (1955). *Arch. exp. Path. Pharmak.*, **225**, 110.
Mendel, B., and Myers, D. K. (1955). *Nature, Lond.*, **176**, 783.
Sawyer, C. H. (1945). *Science*, **101**, 385.
Tabachnick, I. I. A., and Roth, F. E. (1957). *J. Pharmacol.*, in the press.
Vincent, D., and Jullien, A. (1938). *C.R. Soc. Biol., Paris*, **127**, 1506.
Winbury, M. M., Wolf, J., and Tabachnick, I. I. A. (1957). *Fed. Proc.*, **16**, 347.

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TWO KINDS OF TRYPTAMINE RECEPTOR

BY

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(RECEIVED MARCH 6, 1957)

There are two kinds of tryptamine receptor in the guinea-pig ileum, namely the M receptors which can be blocked with morphine and the D receptors which can be blocked with dibenzyline. Atropine, an atropine-like drug, cocaine, and methadone inhibit effects due to the M receptors, even after dibenzyline, but have no additional effect after morphine. Lysergic acid diethylamide, dihydroergotamine and 5-benzyloxygramine inhibit effects due to the D receptors, even after morphine, but have no additional effect after dibenzyline. The M receptors are probably in the nervous tissue and the D receptors are probably in the muscles.

The drugs which act as antagonists of 5-hydroxytryptamine (5-HT) give different results when tested on different preparations. Various derivatives of ergot have been found to be very potent antagonists of 5-HT in experiments on the rat uterus or on the rabbit ear perfused with salt solutions, but the ergot derivatives were not, however, very potent antagonists of 5-HT in the case of the guinea-pig ileum (Fingl and Gaddum, 1953; Gaddum, 1953; Gaddum and Hameed, 1954; Gaddum, Hameed, Hathway, and Stephens, 1955).

In order to explain such facts, Gaddum and Hameed (1954) suggested the existence of two kinds of tryptamine receptor: one in the plain muscle of the rat uterus and the rabbit ear which was easily blocked by lysergic acid diethylamide (LSD), gramine, or dihydroergotamine, and another in the intestine, not easily blocked by such drugs.

There is evidence that 5-HT causes contraction of the guinea-pig intestine by acting through the nerves, but this is not due to an action on the nicotine receptors, since it is not suppressed by hexamethonium or by the eventual paralytic action of large doses of nicotine (Rocha e Silva, Valle and Picarelli, 1953; Robertson, 1953). The tryptamine receptors seem, in fact, to be different from other kinds of receptor, since large doses of 5-HT eventually abolish the effect of 5-HT itself on guinea-pig intestine without diminishing the response to histamine or substance P (Gaddum, 1953) or acetylcholine or nicotine (Rocha e Silva *et al.*, 1953).

Gaddum and Hameed (1954) found that LSD, in a concentration of 0.1 μg./ml., diminished the response of guinea-pig ileum to 5-HT by 50%, but concentrations 100 times larger had no more effect. Cambridge and Holgate (1955) made similar observations with atropine, using histamine and acetylcholine as control drugs. When the responses of guinea-pig ileum to a constant dose of agonist were plotted against the log. dose of atropine these two drugs gave simple S-shaped curves, but with 5-HT there was a plateau over the range of concentrations 0.01 to 1.0 μg./ml. These observations could be explained on the theory that the two types of receptor postulated by Gaddum and Hameed are both present in the guinea-pig ileum and drugs may block one type of receptor without affecting the other type. The results described below confirm this theory and provide a method of studying each type of receptor separately by eliminating the effects of the other type with specific antagonists.

METHODS

Guinea-pig Ileum Preparation.—Pieces of terminal ileum were removed from guinea-pigs (180–250 g.) which had fasted overnight, and suspended in a 2 ml. bath containing aerated Tyrode solution maintained at 35°. The movements of the longitudinal muscle were recorded with a frontal lever having a magnification of ×5.

Effects of Antagonists.—The effects of the antagonists have been measured in terms of the dose-ratio (Gaddum *et al.*, 1955) by constructing dose-effect curves for the stimulant drugs at the beginning of the experiment, and comparing their subsequent effects in the presence of the antagonist with these curves. The dose-ratio is the ratio of equiactive doses in the presence and absence of the antagonist.

The concentration of the various antagonists was maintained in the bath either by adding fresh doses

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whenever the fluid was changed, or by adding the drug to one of the reservoirs from which the bath could be refilled. The effects of most of the compounds used were complete within 30 to 40 min., but some effects were developing even after 60 min. In such cases, 60 min. was adopted as the standard time at which measurements were made. In order to avoid errors due to the persistence of effects, a separate piece of ileum was used for each experiment.

Histamine and nicotine were used as control drugs. The drugs used were: 5-hydroxytryptamine creatinine sulphate [Upjohn]; histamine acid phosphate, atropine sulphate, and nicotine acid tartrate [British Drug Houses]; morphine hydrochloride [Duncan, Flockhart]; dibenzyline [Smith, Kline, and French]; lysergic acid diethylamide (LSD), 2-bromo-lysergic acid diethylamide (BOL-148), and dihydroergotamine methanesulphonate DHErg [Sandoz]; cocaine hydrochloride [Duncan, Flockhart]; 3:3-diphenylpropan-3-ol-1-diethylamine methiodide (186C47) and methadone hydrochloride [Burroughs Wellcome], and 5-benzyloxygramine (5-BOGram) [Glaxo]. Doses are given in terms of the bases.

Results

Compounds were tested on the guinea-pig ileum preparation for their effects against 5-HT, histamine, and nicotine by the following method. Suitable doses of 5-HT (0.01 to 0.100 μg.), histamine (0.005 to 0.015 μg.), and nicotine (0.5 to 1.5 μg.) were added in turn at regular intervals, until regular responses were obtained. The doses were then altered and curves constructed by plotting the height of the responses against the doses. The drugs to be tested as antagonists were added in an initial low concentration (0.001 μg./ml.) and maintained in the bath for 60 min. During this time 5-HT, histamine, and nicotine were reapplied to the intestine and, if necessary, the doses were increased in order to overcome the inhibition caused by the antagonist. If the effects of the stimulant drugs were unchanged, higher concentrations of the antagonists were tried. The dose-ratios were calculated as described above.

Table I shows the results of some experiments with various drugs which antagonize 5-HT. It is included for comparison with Table II, and to show the effects of morphine and dibenzyline alone in the preliminary treatment of the ileum in the later experiments.

Morphine, in a concentration of 0.01 μg./ml., reduced the response to 5-HT by 50 to 70%, but higher concentrations (10 μg./ml.) appeared to have no more effect on it (see Kosterlitz and Robinson, 1955).

The depression of the 5-HT response caused by atropine began with a concentration of 0.001 μg./ml., and increased to 40% at a concentration of 0.01 μg./ml.; no further depression was produced until the atropine concentration was larger than 1 μg./ml. This confirms the results of Cambridge and Holgate (1955).

The same thing, as already observed by Gaddum and Hameed (1954), occurred with LSD. The depression of the 5-HT contraction began with an LSD concentration of 0.001 μg./ml., increased with a concentration of 0.01 μg./ml., but no further depression was observed with much higher concentrations of LSD (10 μg./ml.). The effect of LSD is small compared with its effect against

Table I

RATIOS OF DOSES OF 5-HYDROXYTRYPTAMINE, HISTAMINE, AND NICOTINE CAUSING EQUAL CONTRACTIONS OF GUINEA-PIG ILEUM IN THE PRESENCE AND ABSENCE OF VARIOUS ANTAGONISTS

Antagonist	Conc. (μg./ml.)	Dose-ratio											
		5-HT				Histamine				Nicotine			
Morphine	1	2·3 1·4 5·5 4·0	1·6 1·5 2·6 5·0	8·0 4·1 4·8 2·0	2·6 2·5 4·0 2·6	1·0 1·0 1·0 1·0	1·0 1·1 2·0 1·2	2·5 1·0 1·4 1·2	1·0 1·0 1·6 1·5	4·0 3·3 3·0	1·6 2·1 3·0	2·6 3·7 1·7	3·0 2·2 2·0
Atropine	0·1	3·3	5·0			3·0	1·1			1·3	1·0		
186C47	0·07	2·6	1·4	3·8		1·2	1·0	1·2		2·0	2·5	3·7	
Cocaine	10	50–100	10	20	18	1·2	1·0	1·4	0·8	8·2	2·5	3·0	3·1
Methadone ..	1	50	55			2·0	2·0			10	16		
Dibenzyline ..	0·1	70 60 32 94 53	47 83 30 31	60 40 80 44	33 66 40 41	107 44 150 25	40 85 80 43	40 60 33 100	80 100 210 30	2·3 1·0 2·0 2·0 1·3	1·5 3·0 2·1 1·0	2·2 1·2 1·5 1·2	1·5 1·0 1·6 1·0
LSD	0·01	6·6	8·0			3·0	1·1			1·3	1·0		
DHErg	2·5	52	50			1·2	1·6			2·1	2·0		
5-BOGram ..	0·1	4·0	6·2			1·0	1·0			1·1	1·0		

5-HT observed on the rat uterus (Gaddum *et al.*, 1955). Of the drugs shown in Table I, dibenzyline is the only one with much action as an antihistamine.

After these preliminary experiments, morphine and dibenzyline were chosen as the specific antagonists for the two kinds of tryptamine receptor. The receptors which were blocked by morphine have been called the M receptors and those blocked by dibenzyline have been called the D receptors.

When a concentration of 1 μg./ml. of morphine is maintained in the bath, the M receptors appear to be more or less completely blocked, and any additional antagonistic effect is then thought to be due to an action on the D receptors. When the intestine is exposed to a concentration of 0.1 μg./ml. of dibenzyline for 30 min., the D receptors appear to be more or less completely destroyed and any additional effect is then thought to be on the M receptors. Different pieces of intestine prepared in these two ways thus provide two types of preparation for studying effects of the antagonists on the two types of receptor.

Fig. 1 shows an experiment with atropine (0.1 μg./ml.) on each type of receptor. In the upper part of this figure effects on the M receptors have been eliminated because these receptors were completely paralysed by morphine throughout the experiment. Atropine had very little effect on the response of 5-HT. A dose of 0.06 μg. of 5-HT after atropine had about the same effect as 0.045 μg. of 5-HT before atropine; the dose ratio was therefore estimated as about 1.3. In the lower part of the figure the ileum had been treated with dibenzyline, and the same concentration of atropine had much more effect. It will be seen that 60 μg. of 5-HT after atropine had less effect than 0.2 μg. before atropine. The dose-ratio was therefore greater than 300. These results show that atropine had a large effect on the M receptors and little effect on the D receptors. It had little effect on the response to histamine, and it antagonized the effect of nicotine after dibenzyline less than that of 5-HT.

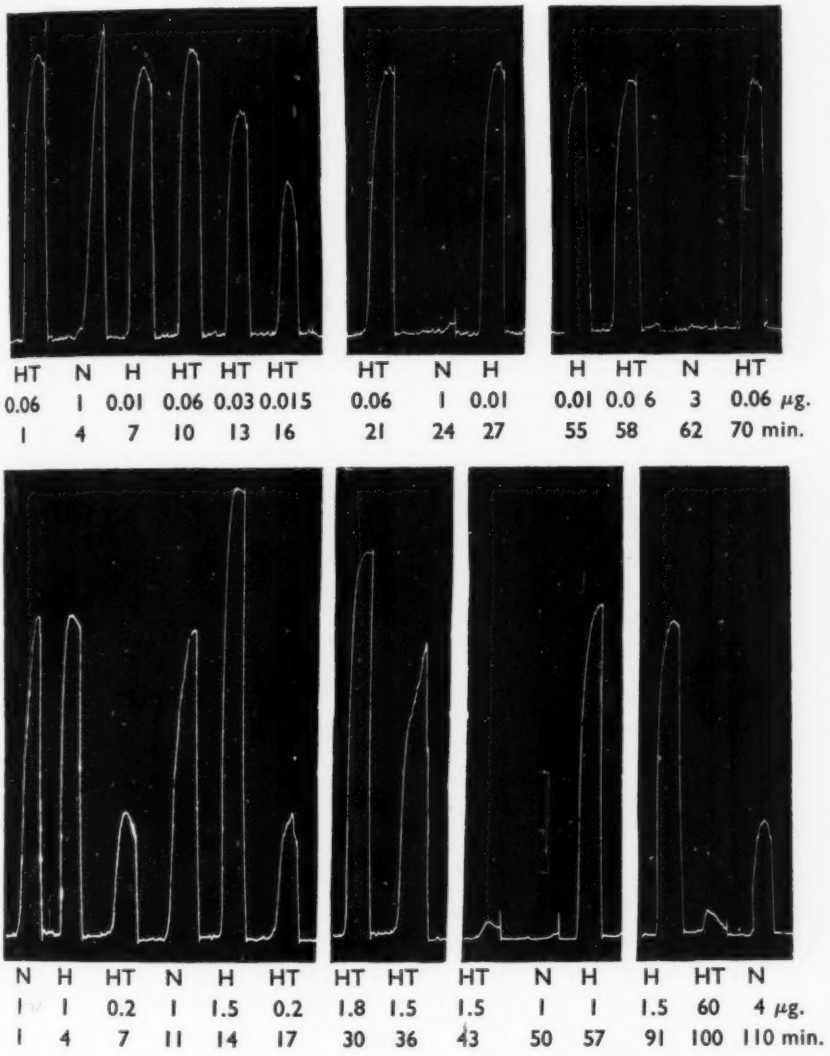

FIG. 1.—Guinea-pig ileum. Contractions due to 5-HT (HT), nicotine (N) and histamine (H). Doses μg. in 2 ml. bath. Above with morphine (1 μg./ml.). Effects on D receptors unchanged by atropine (0.1 μg./ml.) from 18 min. onwards. Dose-ratio = 1.3. Below after dibenzyline (0.1 μg./ml. for 30 min.). Effects on M receptors inhibited atropine (0.1 μg./ml.) from 38 min. onwards. Dose-ratio >300 for 5-HT.

Fig. 2 shows an experiment with 5-benzyloxygramine on each type of receptor. This was the most active of a series of indole compounds tested by Gaddum *et al.* (1955)

for antagonism to the action of 5-HT on the rat uterus. This substance had marked antagonism to 5-HT in the presence of morphine, as shown in the upper part of the figure, where 12 μg. of 5-HT had about as much effect at 97 min. as 0.24 μg. had at 7 min. The dose-ratio was therefore estimated as 50. In the presence of dibenzyline (lower records), the effect of this antagonist was comparatively small; in the lower part of the figure it will be seen that 1 μg. of 5-HT at 110 min. had about the same effect as 0.5 μg. at 4 min. and 16 min. The dose-ratio was therefore estimated as 2.0. These results show that 5-benzyloxygramine had a large effect on the D receptors and a small effect on the M receptors.

The results of such experiments with various 5-HT antagonists are shown in Table II. Here, when the dose-ratio is 1 it means that the second drug has no effect in addition to that produced by the first drug.

The first five drugs have large effects against 5-HT after treatment of the ileum with dibenzyline and little or no effect in the morphine-treated one. They are thought to act on the M receptors.

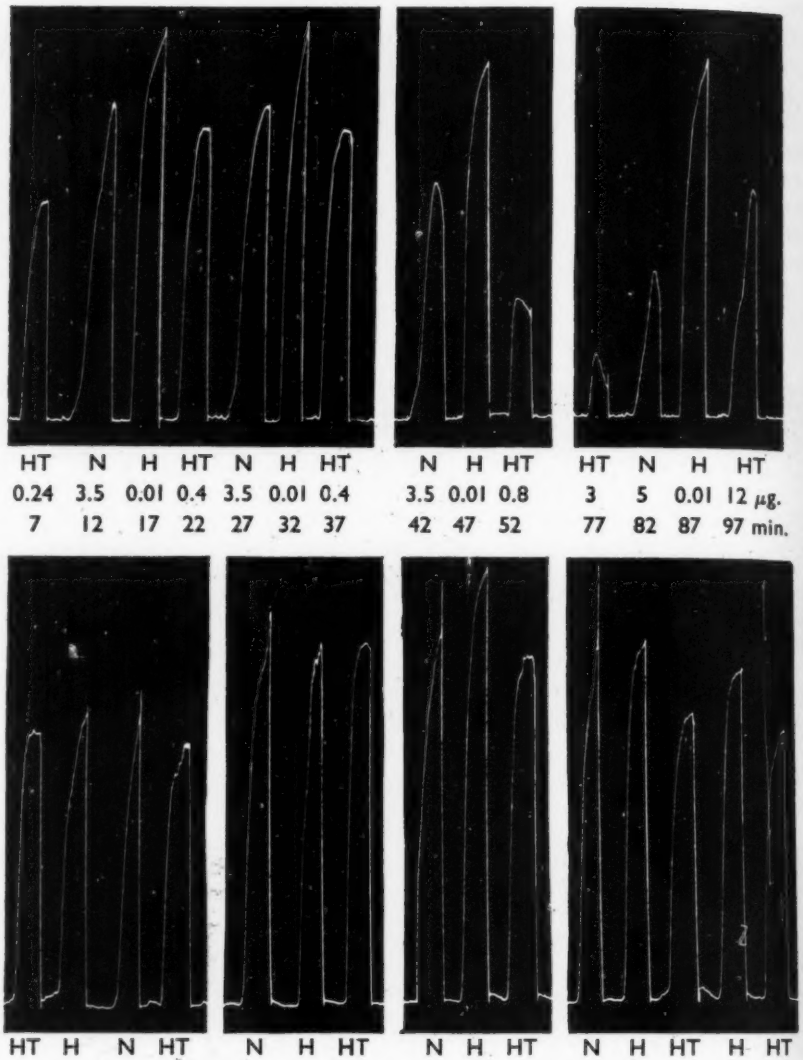

FIG. 2.—Records as Fig. 1. Above with morphine (1 μg./ml.). Effects on D receptors inhibited by 5-benzyloxygramine (1 μg./ml.) from 38 min. onwards. Dose-ratio = 50 for 5-HT. Below after dibenzyline (0.1 μg./ml. for 30 min.). Effects on M receptors almost unchanged by 5-benzyloxygramine from 50 min. onwards. Dose-ratio = 2.

The last five drugs have large effects against 5-HT in the presence of morphine and less action in the ileum previously treated with dibenzyline. They are thought to act on the D receptors.

DISCUSSION

These results indicate that 5-HT causes contractions of the guinea-pig ileum by two different mechanisms, one of which is blocked by dibenzyline (D receptors) and the other of which is blocked by morphine (M receptors).

The drugs which block the D receptors (dibenzyline, ergot compounds, and 5-benzyloxygramine) are the drugs which antagonize the effects of 5-HT on the rat uterus and the rabbit ear. These effects are thought to be due to an action on smooth muscle and it seems probable that the D receptors of the guinea-pig ileum are also in smooth muscle.

The drugs which block the M receptors on the other hand probably act on the nerve ganglia or the nerve fibres. Various authors (Rocha e Silva

TABLE II

RATIOS OF DOSES OF 5-HYDROXYTRYPTAMINE, HISTAMINE, AND NICOTINE CAUSING EQUAL CONTRACTIONS OF GUINEA-PIG ILEUM IN THE PRESENCE AND ABSENCE OF VARIOUS ANTAGONISTS

	Antagonist	Conc. (μg./ml.)	Dose-ratio									
			After Dibenzyline						With Morphine			
			5-HT		Histamine		Nicotine		5-HT		Histamine		Nicotine	
M	Morphine	1	25–35	25–50	1·8		6·6	>10						
	Atropine ..	0·1	>400	>300	1·5	1·0	4–10	3·0	1·0	1·3	1·0	1·0	>3·0	
			>16	>300	3·0	1·0								
	186C47 ..	0·07	>320	500	1·0	2·0	6·0	3·0	1·0	1·4	1·0	1·4	>3·0	>2·0
	Cocaine ..	10	8·0	50–100	1·2	1·5	4·0	>4·0	2·0	1·0	1·0	1·0	6·0	3·0
			10–20		1·0		>4·0		1·0		0·6		>3·0	
	Methadone	1	200	300	2·0	2·0	>4·0		4·0	4·3	1·5	1·6	10	10
D	Dibenzyline	0·1	—		—		—		>400	>500	>400	>1500	>2·5	
	LSD ..	0·01	1·2	6·0	1·0	1·0	2·0	1·0	80–120	75	1·0	1·0	2·6	1·4
			1·0		1·0		1·0							
	DHErg. ..	2·5	2·3	1·5	1·2	1·0	1·1	1·6	100	125	1·2	1·0	1·6	1·6
	5-BOGram.	0·1	2·0	1·3	1·2	1·3	1·0	1·0	50	50	1·2	1·0	1·6	1·6
	BOL-148 ..	0·01	2·0	1·6	1·0	1·0	1·3	1·5	50	80	1·3	1·5	2·6	2·0

et al., 1953; Robertson, 1953; Gaddum and Hameed, 1954; Garven, 1956) have come to the conclusion that 5-HT has an action on nervous structures in the guinea-pig ileum. This conclusion has been based on the similarity between the effects of antagonists on the responses to 5-HT and nicotine, and particularly on the fact that the effects of both these drugs are blocked by cocaine.

The results shown in Table II confirm this conclusion. The first four drugs in the Table all showed marked antagonism to 5-HT after dibenzyline, but no definite antagonism to 5-HT in the presence of morphine. Methadone gave similar results, but did show some effect in the presence of morphine. This may mean that methadone has some action on both types of receptor.

These drugs showed definite but feeble antagonism to nicotine, but no definite antagonism to histamine. Since histamine acts on muscle and nicotine on nervous ganglia, this difference confirms the theory that these drugs act on nervous tissues, but they do not necessarily all act in exactly the same place.

Atropine and 186C47 are known to be specific antagonists of acetylcholine and their effect on the response to 5-HT may be the result of antagonism to the acetylcholine liberated by nerves when these are stimulated by 5-HT. If this is so, it is surprising that their antagonism to the action of nicotine is comparatively feeble. Cocaine is thought to act mainly on the nerve fibres.

The site of action of morphine is uncertain. P. Trendelenburg (1917) found that morphine inhibited both phases of the response of the guinea-pig ileum to a rise of internal pressure, namely the initial contraction of the longitudinal muscle and the subsequent wave of contraction of the circular muscle. O. Schaumann, Giovannini, and Jochum (1952) showed that the actions of various drugs on these internal reflexes are related to their potency as analgesics, so that the study of the actions of these drugs on the intestine may provide a clue to their central actions. Morphine might produce these effects by acting on the nervous ganglia in the intestine. It has been suggested that its site of action may be in the nerve fibres peripheral to the ganglia (W. Schaumann, 1955), but it must be less peripheral than the site of action of atropine, since the action of morphine, unlike that of atropine, is associated with inhibition of the release of acetylcholine (Paton, 1957; W. Schaumann, 1956). These actions of morphine on intestinal reflexes may be related to its antagonism to 5-HT, but the relationship is not necessarily a simple one.

The M receptors for 5-HT are probably in the nervous tissue of the intestine, but it is not possible to say exactly where they are in this tissue. U. Trendelenburg (1956) has found that small doses of 5-HT, histamine, and certain other substances potentiate the action of acetylcholine on sympathetic ganglia, whether it is injected or liberated by nerves. There is some evidence (U. Trendelenburg, 1957) that the actions of the drugs on ganglia are antagonized by morphine. These results suggest by analogy that 5-HT and morphine both act on autonomic ganglia in the intestine, but there is no direct evidence of this and no proof that they act on the same receptors as one another.

Z. P. Picarelli undertook this work during the tenure of a British Council Scholarship.

References

Cambridge, G. W., and Holgate, H. J. (1955). *Brit. J. Pharmacol.*, **10**, 326.

Fingl, E., and Gaddum, J. H. (1953). *Fed. Proc.*, **12**, 320.

Gaddum, J. H. (1953). *J. Physiol.*, **113**, 363.

—— and Hameed, K. A. (1954). *Brit. J. Pharmacol.*, **9**, 240.

—— —— Hathway, D. E., and Stephens, F. F. (1955). *Quart. J. exp. Physiol.*, **40**, 49.

Garven, J. D. (1956). *J. Pharm., Lond.*, **8**, 256.

Kosterlitz, A. W., and Robinson, J. A. (1955). *J. Physiol.*, **129**, 18.

Paton, W. D. M. (1957). *Brit. J. Pharmacol.*, **12**, 119.

Robertson, P. A. (1953). *J. Physiol.*, **121**, 54.

Rocha e Silva, M., Valle, J. R., and Picarelli, Z. P. (1953). *Brit. J. Pharmacol*, **8**, 378.

Schaumann, O., Giovannini, M., and Jochum, K. (1952). *Arch. exp. Path. Pharmak.*, **215**, 460.

Schaumann, W. (1955). *Brit. J. Pharmacol.*, **10**, 456.

—— (1956). *Nature, Lond.*, **178**, 1121.

Trendelenburg, P. (1917). *Arch. exp. Path. Pharmak.*, **81**, 55.

Trendelenburg, U. (1956). *J. Physiol.*, **132**, 529–541.

—— (1957). *Brit. J. Pharmacol.*, **12**, 79.

Brit. J. Pharmacol. (1957), **12**, 329.

THE ACTION OF SUBSTANCES ANALOGOUS TO DIAMINODIPHENOXYALKANES AGAINST *SCHISTOSOMA MANSONI*

BY

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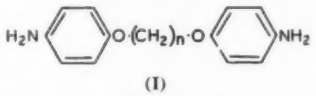
The effects on activity against *Schistosoma mansoni* of further modifications of the diaminodiphenoxyalkanes are reported. Replacement of the amino-group by any of a large number of other groups destroys activity. Activity is retained in the presence of certain substituted (hydroxyalkyl, carboxyalkyl) amino-groups and in some of their aldehyde-bisulphite derivatives. Many variations of the central chain lead to compounds of reduced activity, the outstanding exceptions being a number of but-2-ene derivatives, which retain full activity.

Previous papers from these laboratories (Raison and Standen, 1955; Caldwell and Standen, 1956; Standen and Walls, 1956) have reported the schistosomicidal activities of diaminodiphenoxyalkanes (I) and related compounds. Two further variations of this structure are now considered,



(I)

namely that of the terminal amino-group, apart from mono- and di-alkyl substitution (Raison and Standen, 1955), and that of the aliphatic chain linking the two benzene rings.

MATERIALS AND METHODS

As in the previous papers in this series, the compounds described were tested orally against adult *Schistosoma mansoni* in mice. Treatment commenced 63 days after infection of the mice and was continued twice daily for 5 consecutive days. The animals were killed 7 days after completion of treatment and were examined to determine the drug effects against the parasites. Assessment of results was made according to the methods described by Raison and Standen (1955).

RESULTS

The results of routine screening of those types of compound which showed activity are given in Tables I to IV. The "unit dose" referred to is that given orally twice daily for five days.

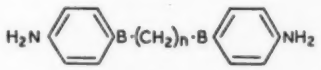
Variation of the Amino-group

Inactive Compounds.—When the amino-groups of (I) were replaced by one of the following groups, inactivity or only slight activity at toxic dose levels resulted: aminomethyl, guanidino, biguanido, amidino, ureido, thioureido, guanidinomethyl, benzylamino, aminoalkylamino, hydrazino, hydroxy, cyanomethylamino, carboxy, azidocarbonyl (.CON_3), nitrosomethylamino, toluene-*p*-sulphonamido, acetamido, ethoxycarbonamido, formyl, succinylamino, and sulphamino. Replacement of the *p*-aminophenyl group by 4-pyridyl or 6- or 8-quinolyl also led to loss of activity.

Active Compounds.—Comparatively few variations led to retention of high activity and the following types are almost the only examples: (i) hydroxyalkylamino (Table I, A), (ii) carboxyalkylamino (Table I, B), (iii) aldehyde-bisulphite derivatives of primary and secondary amines (Table I, C).

Variation of the Aliphatic Chain

Inactive Compounds. — Substances of the general type in which the ether oxygen atoms were omitted, namely diaminodiphenylalkanes (II; B=CH_2, n=7, 8), and those in which the oxygen atoms were replaced by carbonyl (II;



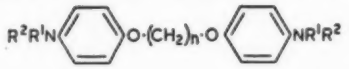
(II)

B=CO, n=7, 8), by ester groups derived either from *p*-aminophenol (II; B=O.CO, n=5) or *p*-aminobenzoic acid (II; B=CO.O, n=5), by

TABLE I

ACTIVITIES OF HYDROXYALKYLAMINES, CARBOXYALKYLAMINES, AND ALDEHYDE-BISULPHITE DERIVATIVES

For explanation of "unit dose" see Results. The doses in section C are those equivalent to 50 mg. of the parent amine



Compound No.	*n*	R^1	R^2	Unit Dose (mg./kg.)	Worms Killed %
A. Hydroxyalkylamines					
221C54	3	$CH_2.CH_2.OH$	H	200 50	5 0
122C54	4	$CH_2.CH_2.OH$	H	100 50	90 57
346C53	5	$CH_2.CH_2.OH$	H	100 50	100 36
347C53	6	$CH_2.CH_2.OH$	H	100 50	96 63
261C53	7	$CH_2.CH_2.OH$	H	50 25 15	100 57 14
262C53	8	$CH_2.CH_2OH$	H	50 25 15	86 47 16
394C53	9	$CH_2.CH_2.OH$	H	100 50	100 70
161C54	10	$CH_2.CH_2.OH$	H	200	0
281C54	4	$CH_2.CH_2.CH_2.OH$	H	200 50	12 0
295C54	5	$CH_2.CH_2.CH_2.OH$	H	200 50	84 0
165C54	6	$CH_2.CH_2.CH_2.OH$	H	200 50	100 43
184C54	7	$CH_2.CH_2.CH_2.OH$	H	50 25	98 0
166C54	8	$CH_2.CH_2.CH_2.OH$	H	200 50	100 68
616C54	9	$CH_2.CH_2.CH_2.OH$	H	50	83
217C54	6	$CH_2.CH(OH).CH_3$	H	50	53
218C54	7	$CH_2.CH(OH).CH_3$	H	50 25 12·5	100 90 21
219C54	8	$CH_2.CH(OH).CH_3$	H	50 25 12·5	98 93 4
483C55	4	$CH_2.CH_2.OH$	CH_3	200 50	19 0
381C53	5	$CH_2.CH_2.OH$	CH_3	100 50	87 11
270C54	6	$CH_2.CH_2.OH$	CH_3	200 50	85 0
271C54	7	$CH_2.CH_2.OH$	CH_3	50 25	88 0
272C54	8	$CH_2.CH_2.OH$	CH_3	200 50	100 35
484C55	9	$CH_2.CH_2.OH$	CH_3	200 50	99 74
265C54	2	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200	0
197C54	3	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200	0
105C54	4	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	93 0
125C53	5	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	100 0

TABLE I—*continued*

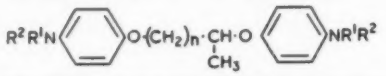
Compound No.	*n*	R^1	R^2	Unit Dose (mg./kg.)	Worms Killed %
197C53	6	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50 25	100 80 0
252C53	7	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50 25	100 90 0
253C53	8	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	50 25	100 12
330C53	9	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	100 34
2C54	10	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	90 0
106C54	11	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200	3
284C54	4	$CH_2.CH(OH).CH_3$	$CH_2.CH(OH).CH_3$	200 50	62 1
287C54	5	$CH_2.CH(OH).CH_3$	$CH_2.CH(OH).CH_3$	200 50	100 0
187C54	6	$CH_2.CH(OH).CH_3$	$CH_2.CH(OH).CH_3$	200 50 25	100 36 0
241C54	7	$CH_2.CH(OH).CH_3$	$CH_2.CH(OH).CH_3$	200 50	93 0
220C54	8	$CH_2.CH(OH).CH_3$	$CH_2.CH(OH).CH_3$	200 50	100 0
454C54	9	$CH_2.CH(OH).CH_3$	$CH_2.CH(OH).CH_3$	200 50	47 0
547C54	8	$CH_2.CH_2.CH_2.OH$	$CH_2.CH_2.CH_2.OH$	200	100
198C54	7	$CH_2.CH(OH).CH_2.OH$	H	50 25 12·5	99 86 1
185C54	7	$CH_2.CH(OH).CH_2.OH$	$CH_2.CH(OH).CH_2.OH$	200	0
B. Carboxyalkylamines					
63C55	4	$CH_2.CO_2H$	H	50	0
13C53	5	$CH_2.CO_2H$	H	50	2
543C54	6	$CH_2.CO_2H$	H	50	96
22C54	7	$CH_2.CO_2H$	H	50 25	96 30
544C54	8	$CH_2.CO_2H$	H	50	90
131C55	9	$CH_2.CO_2H$	H	50	94
167C54	7	$CH_2.CH_2.CO_2H$	H	50 25	92 6
143C55	7	$CH(CH_3).CO_2H$	H	50 25	100 24
358C55	7	$CH_2.CH_2.CH_2.CO_2H$	H	50	98
240C55	5	$CH_2.CO_2H$	$CH_2.CO_2H$	200	0
566C54	7	$CH_2.CO_2H$	$CH_2.CO_2H$	50	87
C. Aldehyde-bisulphite derivatives					
66C55	6	$CH_2.SO_3Na$	H	70	14
398C53	7	$CH_2.SO_3Na$	H	75	89
67C55	5	$CH_2.SO_3Na$	CH_3	75	85
111C55	5	$CH_2.SO_3Na$	$CH_2.CH_2.OH$	85	44
167C55	6	$CH(CH_3).SO_3Na$	H	75	27
236C55	7	$CH(C_6H_5).SO_3Na + NaHSO_3$	H	90	70
168C55	7	$CH(SO_3Na).CH_3—CH(C_6H_5).SO_2Na$	H	125	95
234C55	7	$CH(SO_3Na).(CH.OH)_4.CH_2.OH$	H	110	93

sulphonyl (II ; B=SO_2, n=2–6), or by sulphonamido (II ; B=SO_2NH, n=4, 5, 8, 10 ; B=$NH.SO_2$, n=4), were all inactive at the maximum unit dose of 200 mg./kg. Replacement of the oxygen atoms by imino-groups (II ; B=NH, n=2–6) gave toxic products which were inactive at the maximum tolerated unit dose, which usually did not exceed 20 mg./kg. Insertion of a *p*-phenylene group into the carbon chain destroyed the activity.

TABLE II

ACTIVITIES OF BRANCHED-CHAIN COMPOUNDS HAVING THE FORMULA GIVEN BELOW COMPARED WITH THOSE FOR RELATED STRAIGHT-CHAIN COMPOUNDS

The values given in the last column are those for the compounds with the same total number of carbon atoms in the chain and are taken from Raison and Standen (1955) and from Table I, A.



Compound No.	n	R^1	R^2	Unit Dose (mg./kg.)	Worms Killed %	
					Branched	Straight
115C54	2	H	H	200 50	74 0	99 66
47C53	3	H	H	100 50	96 52	100 78
89C55	3	H	CH_3	200 50	80 —	99 99
64C55	3	H	$CH_2.CH_2.OH$	200 50	97 0	100 36
65C55	3	CH_3	CH_3	200 50	98 1	100 96
91C55	3	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200	39	100

Active Compounds.—The variations of (I) which preserved some degree of activity were: (1) replacement of ether oxygen by sulphur, (2) substitution by alkyl in the carbon chain (Table II), (3) replacement of a methylene group by another atom or group, (4) the introduction of unsaturated linkages into the carbon chain (Tables III and IV).

DISCUSSION

Variation of the Amino Group

The influence of the changes in structure indicated in Table I (A, B, and C) on schistosomicidal activity is best assessed by comparison with the corresponding parent compounds, results for which have already been published by Raison and Standen (1955). Considering first the *hydroxyalkylamines* (Table I, A), the simplest examples are the secondary hydroxyethylamino-compounds ; this series shows the same general picture as the ethylamino-compounds, with a well-marked peak of activity at n=7, but the level of activity is lower. The γ-hydroxypropylamino-series shows a peak at n=7, whereas the propylamino-series had a similar level of activity but with two peaks at n=4 and 7. Only three examples are given of secondary β-hydroxypropylamino-compounds, but their activities are much higher than the corresponding propylamines for the same values of n ; 218C54 and 219C54 have very high activities.

In the tertiary hydroxyethylmethylamino-compounds there are indications of an activity alternating with n, but the level of activity is lower than in the related ethylmethylamines. Bis(hydroxyethyl)-amino-compounds show high activity over the range n=4 to 10 and at the lower dose of 50 mg./kg. a single peak at n=7 to 8 ; the related diethylamines showed an additional peak at n=4 and were in general somewhat more active. The favourable influence of β-hydroxylation of the propyl group is shown again in the tertiary compounds, which show high activity at 200 mg./kg. over the range n=4 to 9, a much higher level of activity than the di-*n*-propylamines. The activity almost disappears at 50 mg./kg., however, making the series less active than their lower homologues, the bis(hydroxyethyl)amines.

Among the *carboxyalkylamines* (Table I, B), the carboxymethylamino-compounds have been most extensively studied. High activity is found in the range n=6 to 9, but in general the compounds are not so active as the parent methylamines. The corresponding esters are much less active. α- and β-Carboxyethyl- and γ-carboxypropyl-amino-compounds have been studied with the single chain length of 7 and high activity was found. It is interesting to compare the activity of the latter compound (358C55) with the related, but inactive, succinylamino-derivative.

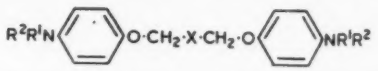
Aldehyde-bisulphite derivatives of a few representative types are shown in Table I, C, in which the doses indicated are equivalent to a dose of 50 mg./kg. of the parent amine. It seems likely that the activity of these derivatives is caused by their hydrolysis, on oral administration, to the original amine.

As far as the amino-group is concerned, therefore, it appears from the results now presented, together with those of Raison and Standen (1955), that very little modification is possible if activity is to be retained. Only alkyl, and such substituted alkyl groups as those described above, are permissible substituents in the primary amines (I). It appears to be essential to retain a group, attached directly to the benzene ring, having the order of basicity of an aromatic amino-group and

TABLE III

COMPARISON OF ACTIVITIES OF RELATED COMPOUNDS HAVING UNSATURATED AND SATURATED CHAINS

The values given in the last column are those for the corresponding saturated compounds of the same chain length and are taken from Raison and Standen (1955) and from Table I, A.



Compound No.	X	R^1	R^2	Unit Dose (mg./kg.)	Worms Killed %	
					Unsaturated	Saturated
A. Compounds with triple bonds						
410C53	C⋮C	H	H	200	0	99
412C53	C⋮C	H	CH_3	200	10	100
533C54	C⋮C	H	$CH_2.CH_2.OH$	200	3	100
467C54	C⋮C	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	0 0	93 0
479C54	C⋮C	CH_3	$CH_2.CH_2.OH$	200	1	19
432C55	$CH_2.C$⋮C	H	H	200 50	88 0	100 78
430C56	$(CH_2)_2.C$⋮C	H	H	200 50	98 4	100 99
271C55	$CH_3.C$⋮$C.CH_2$	H	H	200 50	63 46	100 99
272C55	$CH_3.C$⋮$C.CH_2$	H	CH_3	200	37	100
370C56	$(CH_2)_4.C$⋮C	H	H	200 50	98 44	100 100
273C55	$(CH_2)_2C$⋮C—C⋮$C.(CH_2)_2$	H	H	200 50	39 11	0 —
296C55	$(CH_2)_2C$⋮C—C⋮$C.(CH_2)_2$	H	CH_3	200	0	39
297C55	$(CH_2)_2C$⋮C—C⋮$C.(CH_2)_2$	CH_3	CH_3	200	0	0
B. Compounds with double bonds						
411C53	CH:CH	H	H	200 50	98 18	99 66
8C54	CH:CH	H	CH_3	50 25	100 39	98 36
466C54	CH:CH	H	C_2H_5	50 25	100 24	100 78
311C54	CH:CH	H	n-C_3H_7	50 25	91 2	79 0
465C54	CH:CH	H	$CH_2.CH_2.OH$	200 50	44 0	100 0
480C54	CH:CH	H	$CH_2.CH(CH_3).OH$	200 50	88 8	— —
129C54	CH:CH	CH_3	CH_3	100 50 25	85 57 42	85 60 32
83C55	CH:CH	C_2H_5	C_2H_5	50 25	42 0	35 0
130C54	CH:CH	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	7 0	93 0
478C54	CH:CH	CH_3	$CH_2.CH_2.OH$	200 50	25 2	19 0
131C54	$CH_2.CH$:$CH.CH_2$	H	H	200 50	100 46	100 99
192C54	$CH_2.CH$:$CH.CH_2$	H	CH_3	50 25	100 26	100 90
428C54	$CH_2.CH$:$CH.CH_2$	H	n-C_3H_7	200 50	5 0	66 16

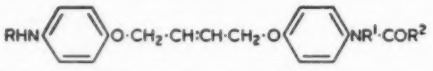
[*continued overleaf*

TABLE III—*continued*

Compound No.	X	R^1	R^2	Unit Dose (mg./kg.)	Worms Killed %	
					Unsaturated	Saturated
468C54	$CH_2.CH:CH.CH_2$	H	$CH_2.CH_2.OH$	200 50	99 0	100 63
236C54	$CH_2.CH:CH.CH_2$	CH_3	CH_3	50 25	80 9	57 0
237C54	$CH_2.CH:CH.CH_2$	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	92 0	100 80
10C54	$(CH_3)_2.CH:CH.CH_2$	H	H	200 50	100 13	100 100
193C54	$(CH_2)_2.CH:CH.CH_2$	H	CH_3	50 25	94 5	100 94
255C54	$(CH_2)_2.CH:CH.CH_3$	CH_3	CH_3	200 50	100 72	100 100
61C55	$(CH_3)_2.CH:CH.CH_2$	$CH_2.CH_2.OH$	$CH_2.CH_2.OH$	200 50	71 2	100 90

TABLE IV

ACTIVITIES OF BUTENE DERIVATIVES CARRYING ONE AMINO GROUP AND ONE URETHANE OR AMIDE GROUP



Compound No.	R	R^1	R^2	Unit Dose (mg./kg.)	Worms Killed %
427C54	H	H	OC_2H_5	200 50	98 64
425C54	H	CH_3	OC_2H_5	50 25	100 67
254C54	CH_3	H	OC_2H_5	200 50	97 58
224C54	CH_3	CH_3	OCH_3	25 12·5	100 85
7C54	CH_3	CH_3	OC_2H_5	25 12·5	95 25
636C55	CH_3	CH_3	$OC_3H_7(n)$	50	98
637C55	CH_3	CH_3	$OC_3H_7(i)$	50	99
541C54	CH_3	CH_3	$OC_4H_9(n)$	50 25	100 39
174C55	CH_3	C_2H_5	OCH_3	50 25	62 17
542C55	CH_3	C_2H_5	OC_2H_5	200 50 25	100 74 0
426C54	CH_3	H	CH_3	50 25	78 41
534C54	CH_3	CH_3	CH_3	25	93

not carrying too large a substituent. The introduction of a more basic group destroys the activity.

Variation of the Aliphatic Chain

Replacement of the ether oxygen atoms by sulphur atoms led to substances either inactive or of much lower activity. Peaks of activity in the series of primary and secondary amines appeared at $n=4$ and $n=9$.

Alkyl (methyl) substitution in the carbon chain was studied in six compounds (Table II), but they proved to be, in general, much less active than corresponding compounds with an unbranched chain of the same number of carbon atoms.

Replacement of one or more methylene groups by an oxygen atom led to compounds the activity of which depended on the position of the substitution. The chain $.O.(CH_2)_2.O.(CH_2)_2.O.$ gave inactive compounds whether the terminal amino-group was primary, secondary or tertiary. With $.O.(CH_2)_3.O.(CH_2)_3.O.$, some activity was found at the highest dose, but with $.O.(CH_2)_4.O.(CH_2)_4.O.$ high activity, comparable with that of the parent diphenoxyalkane, was found. Furthermore, when two methylene groups were replaced by oxygen atoms, as with the chain $.O.(CH_2)_2.O.(CH_2)_2.O.(CH_2)_2.O.$, complete loss of activity resulted. The critical factor appears to be the length of carbon chain between the phenoxy-oxygen and the inserted oxygen atom and these findings may have significance in relation to the speculations of Caldwell and Standen (1956) as to the structural requirements for schistosomicidal activity in this group of compounds. When a methylene group in the chain was replaced by a symmetrically disposed sulphur atom or sulphone group, the compounds were only slightly active.

The fact that replacement of the ether oxygens by any atom or group other than sulphur results in complete loss of activity is consistent with the view that the biological activity of the diphenoxyalkanes is dependent on interaction at this site with a substrate, perhaps by hydrogen bonding, or

even after cleavage of the molecule. Substitution by the sulphur atom, which most resembles oxygen but which forms hydrogen bonds less readily, does not always result in complete loss of activity. Furthermore, the unfavourable effect of branching of the carbon chain at the position adjacent to an ether oxygen, like that of further substitution in the benzene ring (Standen and Walls, 1956), could be attributed to a steric hindrance to substrate-drug interaction.

Unsaturation in the Chain.—The compounds with a triple bond (Table III, A) are all less active than the corresponding saturated compounds, this effect being shown most clearly in the but-2-ynes, which are almost inactive. The distance of the triple bond from the oxygen atoms seems to be important; thus, the primary amines 432C55, 430C56, and 370C56 form a series of increasing activity in which one oxygen is progressively farther away from the triple bond. In the two examples studied (272C55 and 296C55), a methylamino-compound is less active than the corresponding primary amine, the reverse of what was usually found with the saturated compounds.

The comparative inactivity of the but-2-ynes and the low activity of some of the other acetylenes seem to be related to the fact that the triple bond is in the β-position to an oxygen atom. Under certain conditions such compounds are more readily split to yield *p*-aminophenol than are saturated compounds and this was thought to be a possible explanation of the low activity. It was surprising therefore to find that the *trans*-but-2-enes, which are more readily split than the but-2-ynes, are often no less active than the corresponding butanes (Table III, B). In fact, but-2-enes with one terminal basic and one terminal urethane group (Table IV) resemble the saturated compounds of this type (Caldwell and Standen, 1956) in their high level of activity; 224C54 is one of the most active compounds yet investigated. The activity is maintained at a high level for considerable variation of the urethane group, but compounds in which that group is derived from a secondary amine, for example 7C54, are more active than those in which it is derived from a primary amine, namely, 254C54. Replacement of urethane by acetamido (426C54, 534C54) also produced highly active compounds.

Compounds with *trans*-hex-3-ene and hept-3-ene (probably *trans*) chains proved to be usually somewhat less active than the corresponding saturated compounds (Table III, B).

The effect of unsaturation in the chain is interesting in that it does not always decrease activity. Unsaturation imposes a rigidity on the molecule, but in the but-2-ynes and but-2-enes, in which this factor would be expected to be most important, highly contrasting results have been observed. The ease of cleavage of a carbon chain, enhanced by unsaturation, has been discussed above and does not appear to provide an explanation of our results. It is always risky, though tempting, to seek analogies between the *in vivo* and *in vitro* behaviour of drugs. However, the approximate coincidence of the biological results with corresponding saturated and unsaturated compounds (excluding the but-2-ynes) leads one to speculate whether the latter may be hydrogenated *in vivo*. It may then be pointed out that, whereas the but-2-enes described here are smoothly hydrogenated in the presence of various catalysts to butanes, catalytic reduction of the but-2-ynes is much less satisfactory.

We wish to thank the many technical assistants who have contributed to the accomplishment of this work.

References

Caldwell, A. G., and Standen, O. D. (1956). *Brit. J. Pharmacol.*, **11**, 367.

Raison, C. G., and Standen, O. D. (1955). Ibid., **10**, 191.

Standen, O. D., and Walls, L. P. (1956). Ibid., **11**, 375.

Brit. J. Pharmacol. (1957), **12**, 336.

THE EFFECT OF CARRIER STRONTIUM ON THE ABSORPTION OF ORAL DOSES OF RADIOACTIVE STRONTIUM IN RATS

BY

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Carrier strontium had relatively little effect on the retention of an oral dose of radioactive strontium by the rat when it was administered immediately after the radioactive dose. The proportion of the radioactive dose which was excreted in the urine, on the other hand, increased progressively with the carrier dose. There was a decreased uptake of radioactive strontium in rats fed on a special low strontium diet. The effects of dietary strontium are discussed. Evidence was found of a discrimination by the rat against strontium in favour of calcium which was accounted for, at least in part, by a preferential urinary excretion of strontium.

When an oral dose of radioactive strontium is given to experimental animals as a liquid, an appreciable fraction is deposited in the skeleton from which its removal is slow (Hamilton, 1944, 1947; Lisco, Finkel, and Brues, 1947; Kidman, Tutt and Vaughan, 1950). In the form of ^{90}Sr, with a physical half-life of 28 years, radioactive strontium presents a considerable hazard to health and it is not surprising that several attempts have been made to diminish the intestinal absorption and consequent bone retention in experimental animals. Thus, doses of chemical agents or non-radioactive strontium (carrier) have been used (Hamilton, 1944; MacDonald, Nusbaum, Ezmirlian, Barbera, Alexander, Spain and Rounds, 1952; Gross, Taylor, Lee and Watson, 1953), but results from such experiments have not been encouraging. A systematic study of the effect of doses of carrier on the retention of orally administered radioactive strontium, however, has not been attempted.

In the present experiments, the skeletal retention and excretion of a single oral dose of radioactive strontium, ^{89}Sr, has been measured in rats killed 24 hr. afterwards. Different groups of rats received varying amounts of carrier, up to 120 mg./animal, by the same route immediately after the radioactive dose. It has been shown that the absorption of a single dose of strontium from the alimentary canal is approximately proportional to the dose, so that simultaneous administration of oral doses of radioactive and carrier strontium is without any appreciable effect on % of the radioactive dose retained. This result was unaffected by a prolonged experimental period between giving the radioactive dose and the sacrifice of the animals. In addition, the effect of a low strontium diet on the absorption of the radioactive dose has been studied.

METHOD

Male albino rats of a highly in-bred stock were used. These rats were 12 weeks old and weighed about 300 g. They were fed on a stock diet of commercial rat nuts together with water. The calcium, strontium, and phosphorus content of the diet was assayed. Calcium was estimated volumetrically by the permanganate method (Kolthoff and Sandell, 1952), phosphorus colorimetrically by the method of Briggs as modified by Martland and Robison (1926), and strontium by the radioactivation method of Harrison and Raymond (1955). The normal turnover of dietary calcium and strontium was determined for six animals which were kept in metabolism cages on the stock diet with water *ad lib.* Thus the calcium and strontium intake as well as their urinary and faecal excretions were assayed. At sacrifice, the skeletal contents of calcium and strontium were also determined.

Strontium, both radioactive and carrier, was administered by stomach tube under light amylobarbitone anaesthesia. The radioactive dose was 0.1 ml., or about 0.5 μC., from a carrier-free solution of ^{89}Sr. In animals which did not receive carrier, this dose was washed into the stomach with 1 ml. of water while, for

the other animals, the wash was provided by 1 ml. of an aqueous solution of strontium chloride. These animals were killed 24 hr. after the administration of the radioactive strontium.

Urine, including the cage washings, was assayed for radioactivity on an M6H Geiger counter (Veall type) after acidifying with nitric acid. Faeces including the contents of the large bowel were dried and ashed at 650° overnight. The ash was dissolved in nitric acid for counting. Whole skeletons were dissected as free as possible from soft tissue and dissolved in nitric acid. The radioactivity of each sample was corrected for background, paralysis time of the counter and radioactive decay. The error in counting for any specimen was less than 2%. In a few animals sacrificed at the beginning of the experiment, the carcass as well as the alimentary canal down to the caecum was assayed for radioactivity. The activity of both was found to be less than 2% of the total so that these activities were neglected in the later animals. The mean recovery of activity in urine, faeces, and skeleton in 62 animals was 95.3% of the dose with a standard error of ±1.1. The absorbed fraction was regarded as the ratio of the combined urinary and skeletal activities to the total dose. This assessment of absorption appeared to be reasonable since the faecal excretion of parenterally administered strontium is small (Comar, Wasserman, and Nold, 1956; Jones and Coid, 1956).

In order to assess the effect of prolonged excretion on the retention of the radioactive dose with and without carrier, twelve rats were kept in metabolism cages for 12 days after an oral dose of radioactive strontium. Six rats were given the radioactive dose without carrier and six the radioactive dose followed immediately by an oral dose of about 120 mg. of carrier, the administration of the dose and the feeding being as in the shorter-term experiments. Urine and faeces were collected separately and skeletal assays were made at sacrifice of each animal. The effect of a diminished dietary intake of strontium on the absorption of the radioactive dose was investigated following the preparation of a special diet in which the essential nutritive constituents contained little or no natural calcium with its inevitable content of strontium. To this was added repurified calcium carbonate (Analar) to restore the calcium content of this diet to that of the stock diet. The strontium content of calcium carbonate (Analar) which is normally of the order of 200 p.p.m. by weight was reduced some 20-fold by the precipitation of strontium nitrate in concentrated nitric acid followed by the reconversion of the nitrate to calcium carbonate by the addition of ammonium carbonate (Analar). The special diet was analysed for calcium, strontium, and phosphorus by the same methods as those employed for the stock diet.

Five animals were maintained for 21 days on the special diet, by which time it was confirmed that they were in calcium and strontium balance. Another five animals were maintained over the same period on the special diet, to which strontium had been added, to restore the content to that of the stock diet. In this way, any effect of the diet other than the strontium content was eliminated. A carrier-free radioactive dose of strontium was given to both groups and the animals sacrificed 24 hr. later.

Results

Table I gives results of analysis of the stock and special diets for calcium, strontium and phosphorus together with the other main constituents. It will be seen that the diets were similar in essential composition. The stock diet was in the form of extruded, dried nuts while the special diet was fed as a stiff paste, in weighed amounts. No difficulty was experienced in getting the rats to take the special diet.

TABLE I

COMPARISON OF THE STOCK RAT DIET WITH THE SPECIAL DIET

The amount of each constituent is expressed as % by weight. The stock diet was Rat Cake 14% made up by the North Eastern Agricultural Co-operative Society Limited, Aberdeen. For mineral contents of the special diet other than Ca and P see Hansard, Comar, and Plumlee (1951).

Constituent	Diet	
	Stock	Special
Protein	19·2	25·0
Fat	4·9	4·0
Carbohydrate ..	52·6	59·0
Calcium	1·3	1·0
Phosphorus ..	1·0	0·8
Strontium	$2{\cdot}6\times10^{-3}$	$1{\cdot}6\times10^{-4}$
Crude fibre	4·8	—
Water	12·5	8·0
Total ash	6·0	5·4

The results for the strontium and calcium intake and the skeletal content and excretion of these elements for rats fed on the standard diet are given in Table II. Each figure is a mean for six animals

TABLE II

SKELETAL RETENTION AND EXCRETION OF DIETARY STRONTIUM AND CALCIUM/RAT

Mean of six results.

Mineral	Daily Dietary Intake (mg.)	Skeletal Content (mg.)	Excretion	
			Urine (mg./day)	Faeces (mg./day)
Sr ..	0·85	2·66±0·17	0·042±0·014	0·86±0·11
Ca ..	395	3,230±130	14·10±1·97	374±58
$Sr/Ca\times10^3$	2·2	0·82	3·0	2·3

maintained on the diet for at least four weeks before the assays were made. It will be seen that these rats were approximately in strontium and calcium balance. Further, it is to be noted that the skeletal ratio of strontium to calcium was appreciably smaller than the dietary ratio.

Table III shows the effect of doses of carrier strontium from 0 to 120 mg. on the skeletal retention and urinary excretion of a simultaneous oral dose of carrier-free radioactive strontium. The lowest carrier dose was so small that it was not surprising that the mean retention and excretion obtained for these animals was about the same as for those receiving the carrier-free dose. When the carrier dose was increased, the proportion of the radioactive dose which was retained in the skeleton was approximately constant although there appeared to be a slight increase in the value for 5 μg. strontium/g. body weight. The urinary excretion, on the other hand, showed a progressive increase for carrier doses greater than 2 μg./g. body weight.

TABLE III

EFFECT OF SIMULTANEOUS DOSES OF CARRIER STRONTIUM ON THE ABSORPTION OF RADIOACTIVE STRONTIUM

In the last column, % of dose absorbed = % of dose in urine + % in skeleton.

Carrier Sr Added (μg./g. Body Wt.)	No. of Rats	% of Administered Dose (±S.E.)			% of Dose Absorbed (±S.E.)
		Skeleton	Urine	Faeces	
None	9	11·8±1·5	2·5±1·0	80·9±5·3	14·3±2·2
1	5	10·3±2·2	2·0±0·2	82·4±2·7	12·3±2·2
2	5	12·7±3·5	2·4±0·8	78·0±5·9	15·1±4·1
5	6	19·0±3·4	3·4±1·1	70·3±6·1	22·3±3·9
20	5	14·3±4·1	3·6±1·0	74·6±6·6	17·9±5·6
80	5	13·1±2·8	4·6±1·4	77·4±3·1	17·7±3·7
400	5	11·0±1·4	7·1±1·9	71·7±4·9	18·1±2·6

Table IV shows that the skeletal retention of the radioactive dose was unchanged by a simultaneous dose of carrier strontium. In this experiment, it was observed that the urinary excretion was negligible after the first 24 hr. whether carrier was given or not.

TABLE IV

ABSORPTION OF RADIOACTIVE STRONTIUM BY RATS ON THE STOCK DIET SACRIFICED 12 DAYS AFTER AN ORAL DOSE

Mean of six results in each group. See Table III for an explanation of % of dose absorbed.

Oral Carrier Strontium (μg./g.)	% of Administered Dose (±S.E.)			% of Dose Absorbed (±S.E.)
	Skeleton	Urine	Faeces	
None	6·1±0·7	3·4±0·4	89·8±3·3	9·5±1·5
400	7·0±1·0	10·3±0·8	82·8±1·4	17·3±1·8

TABLE V

EFFECT OF A LOW STRONTIUM DIET ON THE ABSORPTION OF AN ORAL DOSE OF RADIOACTIVE STRONTIUM

Mean of 5 results in each group. See Table III for an explanation of % of dose absorbed.

Dietary Sr (mg./Rat/Day)	% of Administered Dose (±S.E.)			% of Dose Absorbed (±S.E.)
	Skeleton	Urine	Faeces	
0·4	9·0 (±3·0)	2·1 (±0·5)	87·1 (±6·6)	11·1 (±3·4)
0·024	5·5 (±3·2)	2·0 (±1·2)	90·0 (±3·7)	7·5 (±4·2)

The results obtained for rats fed on the special diet are shown in Table V. It will be seen that, for the animals fed on the low strontium diet, the skeletal retention, and consequently the percentage of the radioactive dose absorbed, were decreased as compared with litter mates fed on the same diet, to which strontium had been added, to bring the mean dietary intake up to 0.4 mg./rat/day.

DISCUSSION

If the strontium absorbed from the alimentary canal is independent of the amount present, an increase in the amount of carrier strontium would act as an isotopic diluent in the gut and so decrease the absorption of a simultaneously administered radioactive dose. The results given in Table III show that this was not the case. Since the skeletal retention of the radioactive dose is approximately independent of the amount of carrier given, except for the slight increase observed for a carrier dose of 5 μg./g. body weight, the significant increase in the urinary excretion of the radioactive dose with increased carrier must lead to an increased absorption. The small increase in the absorption observed (Table III, last column) is masked by the high skeletal retention at 5 μg./g. already mentioned. Despite a change of 200-fold in the carrier dose, the absorption of the radioactive dose only increased 4%. Over the experimental range studied, it is concluded that the amount of strontium absorbed from the alimentary canal is approximately proportional to the dose.

Since the blood concentrations of strontium following an oral dose depend upon the amount absorbed from the alimentary canal, blood strontium must be temporarily increased. If the renal clearance in the rat is constant, as in humans (Harrison, Raymond and Tretheway, 1955), the rate of urinary excretion should increase with the amount of carrier given. This is in accord with the results given in Table III.

The increased rate of urinary excretion of the radioactive dose compared with that for a carrier-free dose has been cited as evidence of the benefit of carrier as a therapeutic agent following an ingestion of radioactive strontium (Gross *et al.*, 1953). It is clear from the results given in Table III, however, that no such benefit is obtained. The present results were for a 24 hr. period. However, a longer period between the administration of the dose and death of the animals might have revealed a decreased retention of the radioactive dose with carrier due to this increased urinary excretion.

The results given in Table IV show that the skeletal retention of the radioactive dose was independent of the carrier level in the 12 day animals. As already mentioned, in this experiment it was observed that the urinary excretion was quite small 24 hr. or later after the dose whether carrier was given or not. It is quite unlikely, therefore, that prolonging the observation period would have appreciably changed the pattern of the results. The increased urinary excretion of radioactive strontium when carrier is given is thus no indication of a decreased retention.

In view of the fact that the present results show that the overall absorption of strontium from the alimentary canal increased with the amount of strontium given, the effect of diminishing the dietary intake of strontium was deemed to be of special interest. A decrease in the total strontium fed to the animal will produce a decrease in the body pools and, in particular, in the amount of carrier present in the gut. The decreased absorption observed in the animals (Table V) is, therefore, to be expected from the earlier experiments. Recent experiments by Alexander, Nusbaum and MacDonald (1956) have shown that the ratio of dietary strontium to calcium is reflected in the skeletal ratio, animals fed on the higher strontium diet having the higher skeletal ratio of strontium to calcium, a result in agreement with that of the present experiments.

It has been shown independently by Hansard and Plumlee (1954) in the case of calcium, and by Hamilton (1944) for strontium, that a low calcium diet, when fed to rats, leads to an appreciable increase in the absorption of an oral dose of radioactive calcium or strontium. Indeed, Hamilton obtained a 5- to 7-fold increase in the strontium absorbed on a low calcium diet (less than 0.2% Ca). It would appear, therefore, that a low calcium diet produces the opposite effect on strontium absorption from strontium starvation.

From Table II it is seen that the strontium to calcium ratio in the skeleton was 0.82×10^{-3}, whereas the corresponding ratio in the diet was 2.2×10^{-3}, and in the urine 3.0×10^{-3}. The ratio in the urine is thus 1.4 times higher than that in the diet, while that of the skeleton is 2.5 times lower. These figures are in good agreement with the strontium to calcium ratios for the diet, skeleton and urine of rats obtained by Comar *et al.* (1956). It is clear that the kidney of the rat excretes strontium in preference to calcium and that this accounts, at least in part, for the lower strontium to calcium ratio of the skeleton.

References

Alexander, G. V., Nusbaum, R. E., and MacDonald, N. S. (1956). *J. biol. Chem.*, **218**, 911.

Comar, C. L., Wasserman, R. H., and Nold, M. N. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 859.

Gross, W. J., Taylor, J. F., Lee, J. A., and Watson, J. C. (1953). *Univ. of Calif. Atomic Energy Project—ICLA* 259.

Hamilton, J. G. (1944). *U.S. Atomic Energy Commission Report*, MDDC–1001.

—— (1947). *Radiology*, **49**, 325.

Hansard, S. L., Comar, C. L., and Plumlee, M. P. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 455.

—— and Plumlee, M. P. (1954). *J. Nutr.*, **54**, 17.

Harrison, G. E., and Raymond, W. H. A. (1955). *J. Nuc. Energy*, **1**, 290.

—— —— and Tretheway, H. C. (1955). *Clin. Sci.*, **14**, 681.

Jones, H. G., and Coid, C. R. (1956). Ibid., **15**, 541.

Kidman, B., Tutt, M. I., and Vaughan, J. M. (1950). *J. Path. Bact.*, **62**, 209.

Kolthoff, I. M., and Sandell, E. B. (1952). *Textbook of Quantitative Inorganic Chemistry*, 3rd ed., p. 337. New York: Macmillan.

Lisco, H., Finkel, M. P., and Brues, A. M. (1947). *Radiology*, **49**, 361.

MacDonald, N. S., Nusbaum, R. E., Ezmirlian, F., Barbera, R. C., Alexander, G. W., Spain, P., and Rounds, D. E. (1952). *J. Pharmacol.*, **104**, 348.

Martland, M., and Robison, R. (1926). *Biochem. J.*, **20**, 847.

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OXIMES AND ATROPINE IN SARIN POISONING

BY

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Three oximes, mono*iso*nitrosoacetone (MINA), pyridine-2-aldoxime methiodide (PAM) and diacetylmonoxime (DAM), have been examined in combination with atropine as antidotes in sarin poisoning. When treatment was administered 15 min. before sarin, atropine enhanced the protective effect of MINA and DAM 2 to 3 times and of PAM 9 to 10 times in mice and rats. In mice, rats, and guinea-pigs, atropine increased by no more than 2 times the protective effect of all three oximes when given 30 sec. after sarin. Atropine given to monkeys 1 min. after sarin raised the LD50 approximately 3 times. When given in conjunction with MINA or DAM, the LD50 of sarin was raised 7 to 14 times.

It has been shown previously that two oximes, mono*iso*nitrosoacetone (MINA) and diacetylmonoxime (DAM), exert a marked protective effect in rats poisoned with *iso*propyl methylphosphonofluoridate (sarin) (Askew, 1956). Although less active in other species, they still appeared sufficiently promising to justify a further examination in conjunction with atropine. A third oxime, pyridine-2-aldoxime methiodide (PAM), the fastest known reactivator *in vitro* of cholinesterase (ChE) inhibited by sarin (Davies and Green, 1955), was found ineffective against sarin poisoning in rats (Askew, 1956), although active in mice poisoned with di*iso*propyl phosphorofluoridate (dyflos, DFP) or diethyl *p*-nitrophenyl phosphate (paraoxon) (Kewitz, Wilson, and Nachmansohn, 1956). These three oximes have now been examined as adjuvants to atropine in sarin poisoning.

METHODS

The animals used were female mice (18 to 22 g.), rats (180 to 200 g.) and guinea-pigs (320 to 380 g.), and male and female monkeys (2.5 to 4.0 kg.).

The oximes and atropine were given intraperitoneally in aqueous solution, except where otherwise stated. When atropine was given in conjunction with an oxime, the required amount of atropine was dissolved in the oxime solution immediately before use. Sarin was diluted with 0.85% saline and injected subcutaneously in a standard volume for each species. This was 10 ml./kg. for mice, 1 ml./kg. for rats and guinea-pigs, and 0.2 ml./kg. for monkeys.

LD50 values, based on a 24 hr. mortality, were determined both for sarin alone and sarin in the presence of oximes or atropine or both. For each test, the same number of animals was used at each dose, but this number varied in different experiments between four and six animals/group. The ratio between successive doses was 1.26. Since the supply of monkeys was limited, the number used varied at different dose levels, but it was never less than two; here the ratio between doses was 1.3.

LD50 values were normally calculated by the method of moving averages (Thompson, 1947), using the tables constructed by Weil (1952). Where this was not possible the technique of probit analysis (Finney, 1947) was employed. The "relative potency" of an oxime has been expressed as the ratio of the LD50 of sarin in the presence of an oxime to the LD50 of sarin alone. The limits were obtained from the sum of the variances (V) of the individual estimates of the log. LD50, the standard error of the log. difference ($\sqrt{V}$) being multipled by the appropriate value of t at $P = 0.05$ or $P = 0.10$.

In each experiment a control determination of the LD50 of sarin was always made at the same time as that of the inhibitor plus oxime, and these values were the ones used for the calculation of relative potency. Absolute values for the LD50 in control animals varied slightly in different experiments on the same species, but, since in each experiment all dilutions were prepared from the same stock solution, estimates of relative potency are comparable from experiment to experiment.

The two oximes, MINA and PAM, were synthesized at this establishment; DAM was obtained commercially (B.D.H.). They were all more than 95% pure.

RESULTS

The Toxicity of Sarin to Different Species.—Absolute values for the LD50 of sarin to the smaller species are given in Table I, for only

TABLE I

MEAN LD50 VALUES FOR SARIN IN DIFFERENT SPECIES

Sarin in a saline solution was injected subcutaneously.

Species	No. of Animals Used	LD50 Sarin (mg./kg.) Limits $P=0{\cdot}05$
Mouse	80	0·214 (0·119–0·232)
Rat	172	0·116 (0·110–0·121)
Guinea-pig	80	0·046 (0·044–0·049)

values for the relative potency of an oxime are quoted throughout the text. These control values were obtained by combining the mortality figures from each separate estimation of sarin toxicity.

The Effect of Oximes and Atropine in Sarin Poisoning.—MINA (35 mg./kg.), PAM (100 mg./kg), DAM (41 mg./kg. and 150 mg./kg.), and atropine sulphate (50 μM./kg., namely, 17.4 mg./kg.) were given either 15 min. before or 30 sec. after the subcutaneous injection of sarin to mice, rats, and guinea-pigs. The doses of oximes used had previously been found to produce no obvious signs of poisoning in rats (Askew, 1956). The results are shown in Table II.

When MINA and atropine, or DAM and atropine, were given 15 min. before sarin, the protective effect in both rats and mice was significantly greater than when either oxime was given alone. In general, there was a 2- to 3-fold increase in effect. With PAM this increase was considerably more marked. For example, in rats the LD50 of sarin was raised only 1.3 times by either PAM or atropine when used alone, yet together they raised the LD50 approximately 14 times. A similar effect was found in the mouse.

Atropine did not enhance the action of DAM when both were given 30 sec. after sarin, although under the same conditions the protective effect of MINA was slightly increased. When PAM and atropine were given immediately following the injection of sarin, the combined effect was again greater than that of either substance used alone, but the improvement was considerably less marked than when the treatment was administered 15 min. before sarin. In rats, the effect of atropine was enhanced by PAM, although this oxime was ineffective on its own.

When given to guinea-pigs 15 min. before sarin, atropine was found to be without effect in raising the LD50 of the inhibitor, neither did it enhance the action of DAM. This may be due to its breakdown by an atropinase present in guinea-pig liver (Bernheim and Bernheim, 1938). This is consistent with the fact that atropine did exhibit some activity when given immediately following sarin.

The Combined Effect of Oximes and Atropine in Monkeys Poisoned with Sarin.—In the monkey, the oximes and atropine were given by intramuscular injection. Because of the relative insolubility of PAM (approximately 20 mg./ml.), experiments were restricted to MINA and DAM.

MINA proved more toxic to monkeys than to rats. Doses of 25 to 35 mg./kg. intramuscularly caused lethargy accompanied, in many instances, by vomiting. At 20 mg./kg., none of these side effects were obvious and this dose was therefore the maximum used in subsequent experiments. DAM was used in doses of 40 mg./kg. which again produced no obvious toxic signs and which were effective in rats. The dose of atropine (0.029 mg./kg.) was equivalent to 2 mg. to man, which is the dose recommended for use in the treatment of antiChE poisoning (Grob, 1956). Sarin was given subcutaneously into the forearm 1 min.

TABLE II

OXIMES AS ADJUVANTS TO ATROPINE IN SARIN POISONING

Sarin in saline solution was given subcutaneously and all other drugs by the intraperitoneal route. Atropine was administered in a dose of 50 μM./kg. (17·4 mg./kg.). 41 mg./kg. DAM≡35 mg./kg. MINA≡106 mg./kg. PAM.

Interval between Sarin and Other Drug(s)	Oxime	Species	Dose (mg./kg.)	Relative Potency. Limits $P=0{\cdot}05$		
				Atropine Alone	Oxime Alone	Oxime+Atropine
15 min. before	MINA	Rat	35	1·3 (0·96–1·7)	4·6 (3·4–6·3)	11·6 (8·9–15·3)
	PAM	Mouse	100	1·2 (0·87–1·6)	1·4 (1·2–1·7)	11·3 (8·5–15·2)
		Rat	100	1·3 (0·96–1·7)	1·3 (0·96–1·8)	13·8 (9·9–19·2)
	DAM	Mouse	150	1·2 (0·87–1·6)	1·8 (1·4–2·3)	2·5 (2·0–3·2)
		Rat	41	1·3 (0·96–1·7)	5·3 (4·1–6·8)	9·0 (6·3–12·9)
			150		25·8 (19·3–34·4)	48·3 (34·6–67·4)
		Guinea-pig	150	1·0 (—)	2·5 (2·0–3·1)	2·6 (2·0–3·3)
30 sec. after	MINA	Rat	35	1·4 (1·1–1·7)	3·5 (2·6–4·6)	4·8 (3·8–5·9)
		Guinea-pig	35	1·3 (1·1–1·5)	1·7 (1·3–2·0)	2·7 (2·1–3·6)
	PAM	Mouse	100	—	1·3 (0·98–1·6)	1·8 (1·5–2·3)
		Rat	100	1·5 (1·1–1·9)	1·0 (0·79–1·3)	2·1 (1·7–2·5)
		Guinea-pig	100	1·3 (1·1–1·5)	1·8 (1·4–2·2)	3·1 (2·3–4·0)
	DAM	Rat	41	1·4 (1·1–1·7)	4·0 (3·1–5·2)	3·8 (3·0–4·8)
			150		8·3 (6·3–11·0)	8·0 (6·0–10·6)

before the oximes and atropine, which were injected into the thigh. The results are shown in Table III.

TABLE III

THE EFFECT OF ATROPINE ALONE AND WITH MINA OR DAM IN MONKEYS POISONED WITH SARIN

Sarin (0.2 ml. saline/kg.) was injected subcutaneously. Other injections were given intramuscularly 1 min. after sarin. Atropine was given in a dosage of 0.029 mg./0.2 ml./kg.

Treatment	LD50 Sarin (+ Treatment) (mg./kg.) Limits $P=0{\cdot}10$	Relative Potency Limits $P=0{\cdot}10$
None	0·038 (0·027–0·053)	1·0
Atropine	0·114 (0·082–0·156)	3·0 (2·3–4·1)
Atropine + MINA (10 mg./0·2 ml./kg.)	0·255 (0·205–0·332)	6·8 (5·2–9·0)
Atropine + MINA (20 mg./0·2 ml./kg.)	0·524 (0·422–2·16)	14·0 (9·6–20·4)
Atropine + DAM (40 mg./0·67 ml./kg.)	0·330 (0·108–0·571)	8·8 (6–12·7)

After an LD50 dose of sarin, given subcutaneously, signs of poisoning in the monkey appeared within 4 to 6 min., followed about 2 min. later by collapse and a marked decrease in respiratory rate, and death, if it occurred, was usually within 15 min. of the injection.

Although atropine raised the LD50 of sarin by approximately 3 times, recovery in survivors was slow and was not accelerated by the addition of DAM, for, although the combined treatment raised the LD50 about 9 times, the survivors were still in a state of collapse after 6 hr. Atropine combined with MINA (10 mg./kg.) increased the LD50 by almost 7 times and 5 out of 9 survivors were able to stand within 6 hr. of the initial injections. When the dose of MINA was 20 mg./kg., 9 of 11 survivors had reached the same stage of recovery within 3 hr. and the LD50 was raised by approximately 14 times.

DISCUSSION

In general, it has been shown that the protective effect of MINA, PAM, and DAM in sarin poisoning was increased when these oximes are given in conjunction with atropine. Thus, when given 15 min. before sarin, atropine gave a 2- to 3-fold increase in effect over that obtained with either MINA or DAM alone. With PAM, this increase was considerably more marked, the combined action of atropine and PAM being 9 to 10 times greater than that of the oxime itself.

There was a marked reduction in effect both of oximes alone and of oximes and atropine when they were given immediately after the inhibitor. This is probably associated with the very rapid onset of cholinergic signs following subcutaneous sarin. For example, in the rat signs of poisoning appear within 1 min. of receiving 4 times the LD50 dose of sarin, the time to death being approximately 3 min.

Although oximes have been shown to react *in vitro* with antiChE compounds and also to reactivate ChE inhibited by them (Childs, Davies, Green, and Rutland, 1955; Green and Saville, 1956), it is not yet certain how their activity *in vivo* is related to their behaviour *in vitro*.

For example, PAM reacts rapidly *in vitro* with sarin and, in addition, is the fastest reactivator of ChE when inhibited by sarin. It was expected therefore to show a fairly marked activity against sarin poisoning *in vivo*. However, it proved to be considerably less effective than either MINA or DAM in the rat, although the former is slightly less active than PAM *in vitro* whilst DAM only shows approximately one-fifth the activity of MINA.

A possible explanation of this anomaly between the *in vitro* and *in vivo* results may be provided by a consideration of the physical properties of PAM. This oxime is relatively insoluble in water and in contrast to MINA and DAM is likely to be even more lipid-insoluble since PAM, unlike MINA or DAM, cannot be extracted from aqueous solutions by either benzene or chloroform. Thus it is possible that PAM may not be readily distributed to all the main sites of ChE activity in the animal, particularly to the central nervous system.

Death from sarin poisoning in the rat appears to be due to the almost simultaneous development of central respiratory failure and neuromuscular block (Holmes, personal communication). The relative ineffectiveness of PAM when used alone in the rat could therefore be explained if this oxime were unable to penetrate centrally to any great extent. As atropine antagonizes the central effects of antiChE, the marked increase in efficacy of PAM when given together with atropine could be accounted for if the PAM acts peripherally whilst the atropine counteracts the central effects of sarin.

To test the theory that the action of PAM is restricted to peripheral sites only, experiments were carried out by J. P. Rutland, of this department, on the reactivation *in vivo* by PAM of blood and brain ChE of rats poisoned with sarin. When PAM was administered to rats 1 min. after the onset of cholinergic signs following a subcutaneous injection of sarin, reactivation of blood ChE occurred, but no such reactivation of brain ChE could be demonstrated. MINA and DAM, however, do penetrate to the central nervous

system, for quite significant increases in enzyme activity of the brain occur *in vivo*.

In the quest for a suitable antidote to antiChE poisoning, such factors as solubility, stability, and inherent toxicity of the compound must be considered. PAM is relatively non-toxic and can be given in doses up to 100 mg./kg. to the smaller species without apparent harmful side effects. The maximum dose of MINA which can be given to monkeys without producing toxic signs is 20 mg./kg. Nevertheless, with atropine, 10 mg./kg. of MINA will raise the LD50 of sarin about 7 times.

PAM is much less soluble in water than MINA and this makes its use impracticable in larger species at 100 mg./kg., the dose which has been shown to be effective in this work. Tests have not yet been carried out to determine the minimum dose of PAM which will exert a useful effect in sarin poisoning, although Kewitz *et al.* (1956) have shown that somewhat lower doses are effective against dyflos and paraoxon poisoning in mice.

DAM is less toxic than either MINA or PAM and can therefore be given at somewhat higher doses. However, it is only a poor reactivator of ChE inhibited by sarin (Childs *et al.*, 1955), a property known to be of importance in bringing signs of antiChE poisoning under control (Askew, 1956). It is therefore less likely to be of importance than MINA or PAM as a potential antidote to antiChE poisoning.

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References

Askew, Beryl M. (1956). *Brit. J. Pharmacol.*, **11**, 417.
Bernheim, F., and Bernheim, M. L. C. (1938). *J. Pharmacol.*, **64**, 209.
Childs, A. F., Davies, D. R., Green, A. L., and Rutland, J. P. (1955). *Brit. J. Pharmacol.*, **10**, 462.
Davies, D. R., and Green, A. L. (1955). *Trans. Faraday Soc.*, **20**, 269.
Finney, D. J. (1947). *Probit Analysis*, p. 48. London: Cambridge University Press.
Green, A. L., and Saville, B. (1956). *J. chem. Soc.*, 3887.
Grob, D. (1956). *Arch. intern. Med.*, **98**, 221.
Kewitz, H., Wilson, I. B., and Nachmansohn, D. (1956). *Arch. Biochem.*, **64**, 456.
Thompson, W. R. (1947). *Bact. Rev.*, **11**, 115.
Weil, C. S. (1952). *Biometrics*, **8**, 249.

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A SENSITIVE METHOD FOR THE ASSAY OF 5-HYDROXYTRYPTAMINE

BY

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The movements of a strip of fundus from a rat stomach, suspended in a 5 ml. bath at 37°, are recorded by a spring-loaded lever. The muscle is stretched for 15 to 30 sec. between contractions. 1 ng. 5-Hydroxytryptamine (5-HT), 10 ng. acetylcholine or 2 to 10 μg. of histamine all produce contractions, giving tracings about 4 cm. high. In the presence of hyoscine (10^{-7}) low concentrations of 5-HT can be assayed directly from mixtures of the three substances. Sympathomimetic amines interfere with the assay, but this can be overcome. The preparation is robust, potentially suitable for use with an automatic assay apparatus and 10 to 100 times more sensitive than the rat uterus.

The method most widely used for the biological assay of small amounts of 5-hydroxytryptamine (5-HT) is the isolated oestrus uterus of the rat (Erspamer, 1952 ; Amin, Crawford, and Gaddum, 1954). This preparation does not always exhibit the sensitivity and reliability needed for a simple assay procedure and also requires pre-treatment of the rat. During a survey of the effects of drugs on the isolated stomachs of different species (Paton and Vane, unpublished observation), it was noticed that the fundal portion of the rat stomach was singularly sensitive to 5-HT. The method which has been devised makes use of this observation.

METHOD

All doses of histamine and 5-HT, adrenaline, noradrenaline, and isoprenaline (isopropylnoradrenaline) are given in terms of base, all others are in terms of salt. Krebs solution was gassed with 95% O_2+5% CO_2 ; all others were gassed with O_2.

The Preparation.—Rats of either sex were killed by a blow on the head, followed by slitting the throat. The stomach was dissected free from the abdomen and dropped into the bathing solution.

In the first experiments the effect of 5-HT on the rat stomach was observed as a rise of pressure inside the whole isolated organ bathed in Krebs solution. The fundus is a translucent balloon-like tissue, capable of large changes in volume ; the pyloric antrum is thicker and redder and the two parts are separated by a definite ridge on the mucosa. As the contraction was seen to be predominantly of the fundus, the antrum was thereafter removed, except for a band of pyloric tissue wide enough to be tied on to a cannula. The fundal contents were washed away, the cannula tied into place and the muscle suspended in a 40-ml. organ bath at 37°. The fundus was distended with 7 ml. Krebs solution and the internal pressure recorded on a kymograph by a water manometer. Although this method proved too insensitive as an assay procedure, it was possible to compare the relative sensitivity of the fundus with that of the longitudinal or circular muscle. The dose of 5-HT needed to produce a rise of pressure of 1 cm. of water was found. The fundus was then taken off the cannula and cut into a strip, either by cutting spirally around the same plane as the circular muscle, or by opening the fundus along the lesser curvature and cutting to preserve the longitudinal muscle (as shown in Fig. 1). The resultant strips were then hung in a small bath and the movements recorded by an isotonic lever. Whereas the circular muscle strips gave an increase of sensitivity relative to the whole fundus of from 6 to 18 times, the longitudinal muscle gave an increase of sensitivity of 40 to 80 times. For further experiments, the longitudinal muscle was therefore used. It was found convenient still to leave a small band of pyloric tissue attached to the fundus, to act as a marker, and so prevent cutting in the wrong direction after the fundus had been opened into a flat plate. After cotton had been tied to the ends, the strip was gently stretched and any protrusions and fringes of pyloric tissue trimmed away to give a long clean thin strip. One end of the strip was tied to the tissue holder and lowered into the organ bath. The cotton tied to the other end of the strip was attached to the lever.

The Bath.—The organ bath had an internal diameter of 0.85 cm. and was 15 cm. long down to the side arm through which the bath fluid was replenished. With the tissue holder and bubbling tube in place and with gas bubbling through the solution, the bath held 5 ml. of fluid. A rapid stream of gas

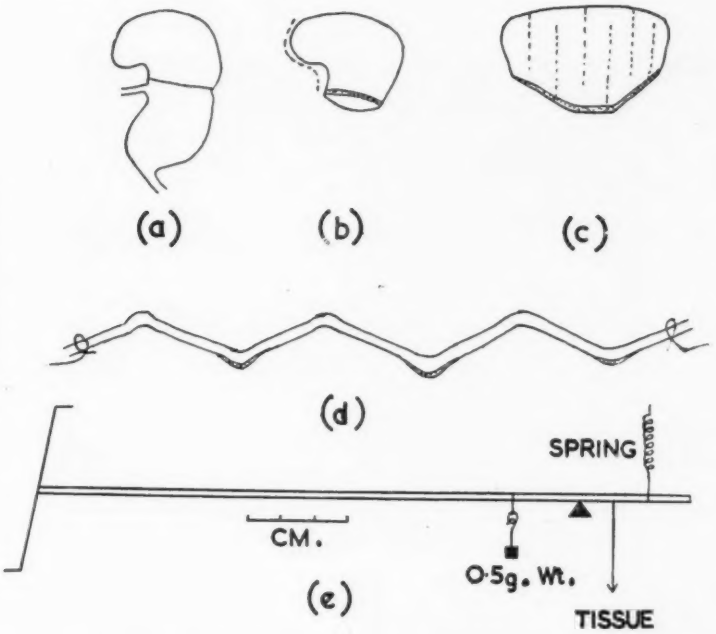

FIG. 1.—Diagram showing the method of cutting the fundal strip and the arrangement of the lever. (*a*), The whole stomach. (*b*), The fundus, left with a thin band of pyloric tissue and then cut along the dotted line. (*c*), The fundus opened into a plate of tissue. Cut along the dotted lines. (*d*), The strip pulled out by cotton tied to each end, so that protrusions and bits of pyloric tissue can be cut off. (*e*), The lever, showing the relative positions of the load, the tissue and the spring. Without any of these, the lever should come to rest horizontally. The scale refers to the lever only.

was used to ensure good stirring. With such a small bath, there was a high ratio of tissue to bathing solution, and if the solution was left unchanged for more than 6 to 8 min. the tissue started to contract. Presumably this was due to the accumulation of metabolites in the bathing fluid, since it could easily be reversed by replacing the solution. To prevent this effect, whenever the preparation was left unattended for more than 5 min., the bathing solution was allowed to perfuse through the organ bath at a rate of about 1 ml./min. A similar slow perfusion was also used immediately after the strip had been isolated so that it could be left to relax for 10 to 15 min.

The Lever.—The lever was made from balsa wood and had a frontal writing point. Because the tissue has little or no spontaneous rhythm, the relatively high magnification of 16:1 could be used. With a purely isotonic lever it was difficult to maintain a steady baseline, but this drawback was partially overcome by having a constant load of 1 g. relative to the tissue coupled with a very light spring, so that as the muscle contracted the restoring force increased. A spring of resting length 3 cm., extending 3.2 cm./g. load, was attached to the lever at a point twice the distance of the muscle from the fulcrum. It was extended to about 5 cm., thus providing a further resting load of about 1 g. with a slight increase in load as the muscle contracted (Fig. 1). A vibrator was attached to the lever holder to reduce the friction of the writing point.

RESULTS

Response to Drugs.—The contractions produced by 5-HT or acetylcholine were sluggish, taking up to 3 min. to achieve a maximum. Fortunately about 90% of the contraction was over in 1½ to 2 min. so that it was possible to obtain reproducible effects by leaving the drug in contact with the tissue for 90 sec. The relaxation of the muscle, especially after high doses such as 5 ng. of 5-HT (10^{-9}), was slower still, sometimes taking 6 min. to return to the former baseline. This potential disadvantage was eliminated when it was found that stretching the muscle to a fixed length for 15 to 30 sec. after each contraction gave a satisfactory baseline and allowed the drugs to be added once every 4 min. (Fig. 2). The following cycle was used: the drug was added to the bath and the contraction allowed to develop for 90 sec. The bath was then washed out by overflow and a weight added to the lever so that it came to rest immediately on a bar fixed at a predetermined level. After a constant time (15 to 30 sec. depending on the individual muscles) the extra weight was removed. The tissue rapidly regained its basal length and after a further 2 min. the next dose was added. The technique can be inferred from Fig. 2.

Sensitivity to Drugs

5-Hydroxytryptamine, Acetylcholine and Histamine.—One of the tracings reproduced as Fig. 2 was obtained from a tissue of average sensitivity; the other from one of the most sensitive preparations, in which a measurable response to 50 pg. of 5-HT (a concentration of 10^{-11}) was recorded. Even the least reactive of the 18 strips tested responded to 1 ng. 5-HT (2×10^{-10}). Fig. 3 com-

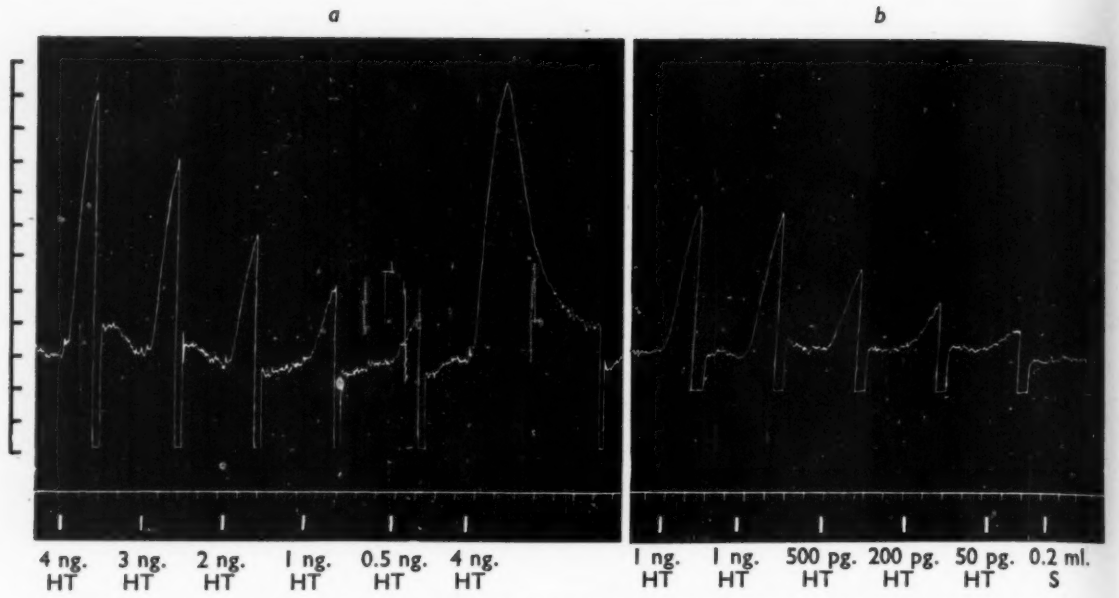

FIG. 2.—(*a*), The response of the fundal strip to doses of 5-hydroxytryptamine (HT) in nanograms (ng.). The drum was left running continuously to illustrate the cycle. The second dose of 4 ng. HT shows the slowness of relaxation if the tissue is not stretched. (*b*), A more sensitive preparation showing responses down to 50 picagrams (pg.) HT, with an injection of 0.2 ml. saline at the end. For this and subsequent figures, a 5 ml. bath containing rat uterus Ringer at 37° was used. The lever, of magnification 16: 1, is described in the text. The bath is washed out by overflow at the beginning of each downstroke. The vertical scale is in cm. Time in min.

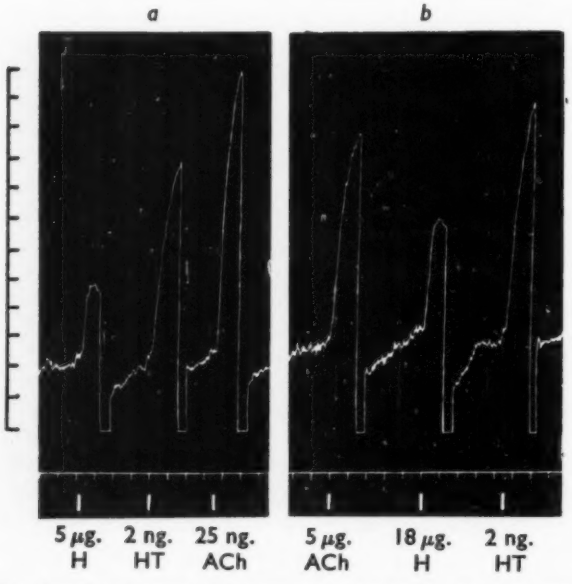

FIG. 3.—The effects of 5-hydroxytryptamine (HT), histamine (H), and acetylcholine (ACh) on the fundal strip before and after hyoscine hydrobromide. (*a*), Contractions produced by 5 μg. histamine, 2 ng. HT and 25 ng. acetylcholine. Hyoscine was added to the Ringer to give a final concentration of 10^{-7}. (*b*), Shows that sensitivity to acetylcholine is greatly reduced; 5 μg. acetylcholine and 18 μg. histamine now have to be used. The response to 2 ng. HT is slightly larger than before. Scale and time as in Fig. 2.

pares the reaction of the fundal strip to 5-HT, acetylcholine and histamine. The sensitivity to acetylcholine was usually about 1/10th of that to 5-HT; histamine had to be given in μg. doses to produce any effect at all. In order to render the muscle insensitive to acetylcholine, hyoscine hydrobromide (10^{-7}) was added to the bathing fluid. Hyoscine was used in preference to atropine because Paton and Rosales (personal communication) have shown it to be the more specific. The second part of Fig. 3 shows that the response to 5-HT (2 ng.) was unaffected by hyoscine, but that acetylcholine and histamine only produced responses in doses several thousand times greater.

Other Drugs.—Several other drugs also induce a contraction in this preparation, and to give a rough comparison of their potencies, the dose was estimated which produced a similar contraction to that given by 1 ng. 5-HT. For instance, nicotine acid tartrate (25 μg.) gave a contraction equivalent to that produced by 1 ng. 5-HT, but after hyoscine (10^{-7}) the same dose of nicotine caused a relaxation of the tissue. The equiactive doses of the other drugs tested are given below, all in the presence of hyoscine. Adenosine triphosphate (20 μg.), amphetamine sulphate (3 μg.), barium chloride (30 μg.), bradykinin (1 to 2 μg.), pitressin (Parke, Davis) (0.4 units), potassium chloride (2

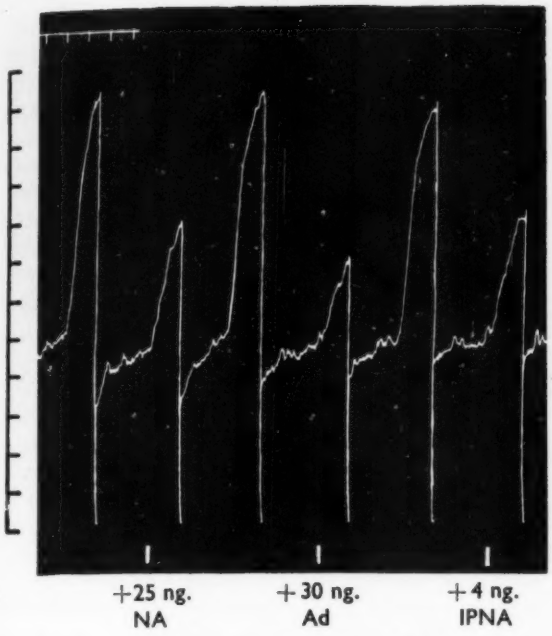

FIG. 4.—This shows the effects of sympathomimetic amines on contractions of the fundal strip produced by a standard dose of 5 ng. 5-hydroxytryptamine (HT). The contractions are reduced by the simultaneous addition of 25 ng. noradrenaline (NA), 30 ng. of adrenaline (Ad) or 4 ng. isopropylnoradrenaline (IPNA). Drum turned off during application of stretch. Scale and time as in Fig. 2.

mg.), tryptamine hydrochloride (250 ng.). Oxytocin solution (containing chlorbutol) caused a relaxation. 5-Hydroxyindole acetic acid had no effect in doses of up to 100 μg.

It will be noted that amphetamine contracted the fundic strip, but most of the sympathomimetic amines relaxed the tissue, or reduced the effects of 5-HT. Isoprenaline was the most active in this respect, with adrenaline and noradrenaline giving similar effects in doses five times larger (Fig. 4).

Comparison of Various Bathing Solutions

The reactions of rat fundal strips were compared in Krebs solution, Tyrode solution and in the rat uterus Ringer and rat colon Ringer used by Gaddum, Peart and Vogt (1949). In both Krebs and Tyrode solution the muscle was slightly more sensitive to 5-HT, but the spontaneous movements were greater than in rat uterus Ringer solution. Changing to rat colon Ringer, in which the calcium and bicarbonate are low, greatly reduced the sensitivity to 5-HT. This was due to the low calcium concentration, since the addition of bicarbonate had no effect, whereas the sensitivity returned to that in rat uterus Ringer when calcium was added. The addition of magnesium

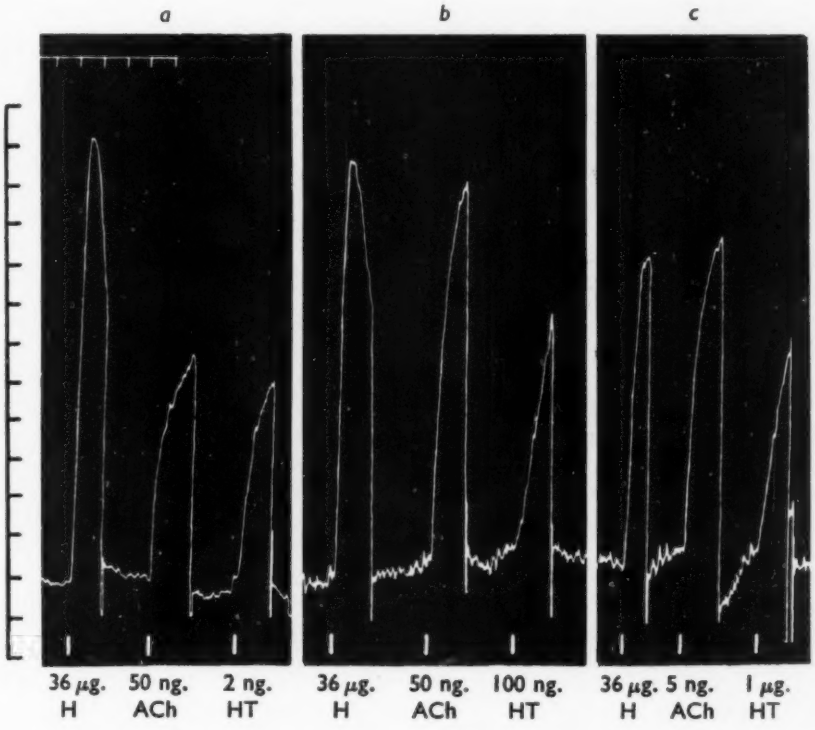

FIG. 5.—The antagonism of 5-hydroxytryptamine (HT) by brom-LSD on the fundal strip. Contractions produced by 36 μg. histamine (H), 50 ng. acetylcholine (ACh) and different doses of HT. (*a*), Before brom-LSD, 2 ng. HT gives a good contraction. (*b*), With 0.2 μg./ml. brom-LSD in the bath, preparation is more sensitive to ACh but is only 1/50th as sensitive to HT. (*c*), With 1 μg./ml. brom-LSD in the bath, the histamine effect is beginning to be depressed; but now 1 μg. HT gives a comparable contraction to 2 ng. HT in (*a*). Drum turned off during application of stretch. Scale and time as in Fig. 2.

to rat uterus Ringer to give double the concentration in Tyrode solution also reduced the sensitivity.

Temperature

37° was found to be the best temperature for this preparation. Lower temperatures made the muscle less sensitive, while higher temperatures elicited greater spontaneous movement.

Antagonists

It has already been shown that hyoscine (10^{-7}) does not antagonize the contraction produced by 5-HT. Morphine sulphate in concentrations up to 2×10^{-5} was also found to have no antagonistic effect. On the other hand, both 2-bromo-lysergic acid diethylamide (brom-LSD) and dibenyline were found to be good antagonists to 5-HT on this tissue. The effects of the antagonists developed over 60 to 90 min. and were not easily reversed. The effects of acetylcholine, histamine, barium chloride and bradykinin were unaltered by either brom-LSD (up to 10^{-6}) or dibenyline (10^{-7}) whereas the effect of 5-HT was greatly reduced (Fig. 5).

Samples of rabbit and cat serum, taken without special precautions, were assayed for their 5-HT content. They were found to contain the equivalent of 2 μg. and 1 μg. 5-HT/ml. respectively. The activity of the serum was reduced by brom-LSD to the same degree as was that of 5-HT.

A high dose of tryptamine desensitized the tissue to 5-HT for 30 min. A similar desensitization, but for a much shorter time, followed a high dose of 5-HT (10^{-8}). This antagonism was short-lived and only applied to the subsequent contraction.

Another facet of this effect was seen if the interval between doses was decreased. As the dose interval became shorter, so the contraction produced by a constant dose of 5-HT became smaller. The regime of a contraction every 4 min. represents a compromise between developing desensitization and the need for the assay to be speedy.

Assay Precision

Fig. 2 gives an idea of the discrimination of the tissue between different doses of 5-HT. Whether a method of bracketing between known doses is used, or some other method to which statistical calculation can be applied, depends on the preference of the user. A mixture of acetylcholine, histamine, and 5-HT (30 ng./ml. acetylcholine, 100 ng./ml. histamine, 7 ng./ml. 5-HT) the contents of which were unknown to assessor was assayed to contain between 6.7 and 8.1 ng. of 5-HT/ml. by the method of bracketing the unknown between known doses.

Discussion

The selective sensitivity of the rat fundal strip to 5-HT makes it a very suitable preparation for the assay of this substance in tissue extracts and biological fluids. Other substances which may act as contaminants were tested, but most of them had to be used in concentrations many times greater than are likely to appear in such extracts. Indeed, the only substances found which may interfere with the assay of 5-HT were the sympathomimetic amines, and if their presence is suspected in the extract, they can be differentially destroyed by a procedure such as that used by Garven (1956).

The greatest difficulties in using this preparation have arisen from the slowness of the relaxation after a dose of 5-HT and from changes in the resting length of the muscle. The latter is only to be expected when it is realized that the contracted length of the strip is about 6 cm., but that the working length under the conditions described is 12 to 15 cm. Furthermore, a change in length of 1 mm. in the muscle is recorded as a change of 16 mm. on the drum. It was surprising that the manœuvre of stretching the muscle after a contraction not only solved the problem of the slowness of relaxation, but also contributed greatly to the production of a fairly stable baseline. That this sudden stretch did not appear to damage the tissue is perhaps due to the natural capacity of the stomach to deal with such emergencies. The maintenance of a stable baseline, however, is only a matter of preference, for the reaction of the strip to 5-HT remains substantially the same over a wide range of resting lengths of the muscle.

It follows, from what has been said above, that most of the difficulties encountered have been a direct consequence of making the method one of high sensitivity using only the bottom part of the dose/response curve. Under these conditions it will detect as little as 50 pg. injected into the bath, or if the whole of the bath fluid can be replaced with a similar solution containing the 5-HT to be assayed, the concentration of 10^{-11}. If even higher sensitivity is required, then it may well be worth while trying the technique of superfusion described by Gaddum (1953), or changing the load on the lever. On the other hand, if such a high sensitivity is not required the preparation becomes easier to handle. Halving the magnification of the lever, halving the length of the strip and doubling the size of the bath will reduce the sensitivity to one-eighth, so that a measurable contraction will now be obtained with from 1 to 4 ng. of 5-HT. If this is done then it may prove possible to elim-

inate some of the steps which were taken to maintain a constant baseline in the face of high sensitivity. The choice of bathing solutions depends upon the nature of the extract to be assayed, but if this is not a limiting factor, then rat uterus Ringer gives the least spontaneous movement.

The main advantages of this preparation over the rat uterus are that it does not need pre-treatment of the donor rat, that it will work in several bathing solutions, and that it can always be relied upon to respond to 1 ng. 5-HT, and very often to 0.1 ng. 5-HT, added to the 5 ml. bath.

I would like to thank the technical staff for their assistance, and Miss D. Armstrong for a small sample of bradykinin.

References

Amin, A. H., Crawford, T. B. B., and Gaddum, J. H. (1954). *J. Physiol.*, **126**, 596.

Erspamer, V. (1952). *Ric. sci.*, **22**, 694.

Gaddum, J. H. (1953). *Brit. J. Pharmacol.*, **8**, 321.

—— Peart, W. D., and Vogt, M. (1949). *J. Physiol.*, **108**, 467.

Garven, J. D. (1956). *Brit. J. Pharmacol.*, **11**, 66.

Brit. J. Pharmacol. (1957), **12**, 350.

THE ACTION OF HYPOGLYCAEMIC SULPHONYLUREAS ON CARBOHYDRATE METABOLISM IN THE FASTED RAT

BY

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Tolbutamide and carbutamide given orally to fasted rats cause a rise in the liver glycogen content 1½ to 3½ hr. after administration of the drugs. Glycogen accumulates preferentially in the right lobe. Subcutaneously injected tolbutamide has the same effect. Both sulphonylureas cause inhibition of glucose-6-phosphatase activity of rat liver homogenates *in vitro*, but at drug concentrations comparable with those found in plasma of treated patients the degree of inhibition is less than 10%. Livers from treated rats show normal glucose-6-phosphatase activity. The glucose uptake of the isolated rat diaphragm is unaffected by the sulphonylureas added *in vitro*. Diaphragms from treated rats show normal glucose uptake in the presence or absence of insulin. The inferences to be drawn from these results are discussed in the light of previous work. It is concluded that the sulphonylureas exert their hypoglycaemic action by inhibiting glycogenolysis and it is suggested that they might do so by inhibiting release of glucagon from the pancreas.

Various theories have been put forward to explain the hypoglycaemic action of the sulphonylureas, N-(*n*-butyl)-N′-(*p*-aminophenyl)-sulphonylurea (BZ55 or carbutamide) and N-(*n*-butyl)-N′-(*p*-methylphenyl)-sulphonylurea (D860, Rastinon or tolbutamide). These theories, summarized recently by Levine and Duncan (1956), have suggested the following possible modes of action: 1. Stimulation of insulin production in the β cells of the pancreas. 2. Inhibition of insulin destruction in the liver, and hence potentiation of its action. 3. Inhibition of glucagon production in the pancreas. 4. Suppression of glycogen breakdown in the liver by inhibition of one or other of the enzymes of the glycolytic system. 5. Inhibition of gluconeogenesis. Much experimental evidence has been quoted in favour of each of these hypotheses, but none of them has been generally accepted. Essentially, they postulate that the sulphonylureas affect either the central mechanisms in the liver controlling carbohydrate metabolism or the utilization of carbohydrate in the peripheral tissues. The experiments to be described were designed to determine in which of these two most likely sites the sulphonylureas exert their main action.

METHODS

Rats.—Male albino Wistar rats weighing 130 to 230 g. were fed *ad libitum* on a standard rat cake diet. They were then starved for 24 hr., but had free access to water. At the end of this time they were weighed and killed by decapitation.

Sulphonylureas.—For oral administration with a stomach tube, carbutamide was suspended in water, and tolbutamide dissolved in 3% aqueous sodium carbonate. Tolbutamide was also given subcutaneously in 10% aqueous solution (*p*H 8.9). For either route of administration the drugs were given in a dose of approximately 500 mg./kg. body weight, the animals being killed 1½, 2½, or 3½ hr. later. Control animals received 3% sodium carbonate (2 ml.) orally, or physiological saline (2 ml.) subcutaneously, whichever was appropriate.

Blood Sugar Estimation.—The blood sugar concentrations of all rats were determined in samples obtained from the neck at the time of decapitation. A modification of the Schaffer-Hartmann method as described by King (1951) was used throughout for glucose estimation.

Rat Diaphragm.—Glucose consumption by the isolated diaphragm was determined by the method described by Vallance-Owen and Hurlock (1954), with the exception that Krebs-Ringer bicarbonate buffer was used as the medium for soaking and incubating the muscle instead of the buffer described by Gey and Gey (1936). Glucose consumption during incubation for 90 min. is expressed as μg. glucose/100 mg. dry weight of muscle.

Liver Glycogen Estimation.—Either the whole liver or portions (0.5 to 1.0 g.) of the right median and left lobes were removed and placed in hot 30%

aqueous KOH within 60 sec. of the time of decapitation. The glycogen content, as determined by the method of Good, Kramer, and Somogyi (1933), is expressed as g. glucose/100 g. wet weight of liver, or as total liver content in mg. glucose/100 g. of rat body weight.

Glucose-6-phosphatase Activity.—This was estimated by a modification of Swanson's method (1950). Immediately after death the liver was removed and cooled rapidly to approximately −10°, such tissue being used immediately or kept frozen at −15° for subsequent use up to two weeks later; activity was found to be unaffected by storage under these conditions. Portions (0.3 to 0.5 g.) were homogenized in 20 vol. of ice-cold 1% aqueous glucose solution. and the homogenates used immediately. Glucose-6-phosphate solution (0.04 M) was obtained by shaking 80 mg. of the barium salt (L. Light) with 2 ml. of an ion-exchange resin (Zeo-Karb 225, sodium form) suspended in 5 ml. of maleate buffer (0.1 M, *p*H 6.3) for 10 min. Tolbutamide and carbutamide, when added *in vitro*, were dissolved in the maleate buffer before addition to the incubation mixture.

To 0.3 ml. of the liver homogenate and 0.6 ml. of the maleate buffer, with or without added sulphonylurea, was added 0.3 ml. of glucose-6-phosphate solution. Using the method originally described by Ernster, Zetterström, and Lindberg (1950), and later modified by Strickland, Thompson, and Webster (1956), the inorganic phosphate content of this mixture was determined immediately after addition of the glucose-6-phosphate and again after 30 min. incubation at 37°. Glucose-6-phosphatase activity is expressed as mg. inorganic P released/g. wet weight of liver/30 min.

RESULTS

Rat Diaphragm.—If the sulphonylureas have any direct peripheral action one would expect to be able to show an effect *in vitro* upon glucose consumption by the isolated rat diaphragm. When added to the incubation medium in concentrations of 50 mg./100 ml., neither tolbutamide nor carbutamide exerted any appreciable effect (Table I); glucose consumption by one hemidiaphragm incubated in the presence of the drug was compared with that of its partner which was used as a control. Clarke, Davidson, Schonbaum, and Serman (1956) had suggested that glucose consumption in the rat diaphragm might be inhibited by carbutamide. The present results are not in agreement with their conclusions and contraindicate any direct peripheral action by these drugs; on the other hand, they do not eliminate the possibility that the sulphonylureas act indirectly on peripheral tissues by increasing insulin secretion *in vivo*. To test this latter possibility tolbutamide was given 2 hr. before the animals were killed, but again glucose consumption by the diaphragms of treated rats incubated in the presence or absence of insulin (10^{-3} i.u./ml.) was the same as that of the controls (Table II).

TABLE I

EFFECTS OF TOLBUTAMIDE AND CARBUTAMIDE (50 MG./100 ML.) ON GLUCOSE CONSUMPTION OF THE ISOLATED RAT DIAPHRAGM

Mean values ± standard error.

Drug	No. of Rats	Glucose Consumption μg./100 mg. Dry Wt.	
		Control Hemidiaphragm	Test Hemidiaphragm
Carbutamide ..	8	263 ± 6	244 ± 19
Tolbutamide ..	11	323 ± 9	359 ± 12
.. ..	8	289 ± 12	274 ± 23

TABLE II

EFFECT OF TOLBUTAMIDE (500 MG./KG.) 2 HR. BEFORE DEATH UPON GLUCOSE CONSUMPTION OF THE ISOLATED RAT DIAPHRAGM

Mean values ± standard error.

	Glucose Consumption (μg./100 mg. Dry Wt. of Muscle)	
	Hemidiaphragm Without Insulin	Hemidiaphragm + Insulin 10^{-3} i.u./ml.
Controls (7) ..	303 ± 13	612 ± 33
Tests (8)	310 ± 13	649 ± 9

That the sulphonylureas do not act peripherally is also suggested by the fact that, in a few experiments which were carried out to study the action of tolbutamide *in vivo*, no rise in muscle glycogen was found 2½ hr. after administration of the drug; this is in accordance with the results of Miller and Dulin (1956) and of Beringer and Lindner (1956).

Liver Glycogen. — Several workers have described the effect of tolbutamide upon the glycogen content of the liver. Bander and Scholz (1956) noted that it had a sparing action on liver glycogen in fasting guinea-pigs, a finding which Tybergheim, Halsey and Williams (1956) and Lang and Sherry (1956) confirmed in rats. Miller and Dulin (1956) observed a rise in liver glycogen content in fasted rats killed 8 hr. after tolbutamide, and a similar effect was produced (Beringer and Lindner, 1956) in fasted rabbits 2½ hr. after carbutamide.

These reports suggest that the sulphonylureas might lower the blood sugar by inhibiting glycogenolysis in the liver. If this is so, the rise in liver glycogen might be expected to occur in the first few hr. after administration of the drugs, in close relation to the fall in blood sugar. Of the experiments quoted above, only those of Beringer and Lindner (1956) refer to this early period. In order to investigate this point further, rats were killed at intervals in the first 3½ hr. after a standard dose of the sulphonylurea.

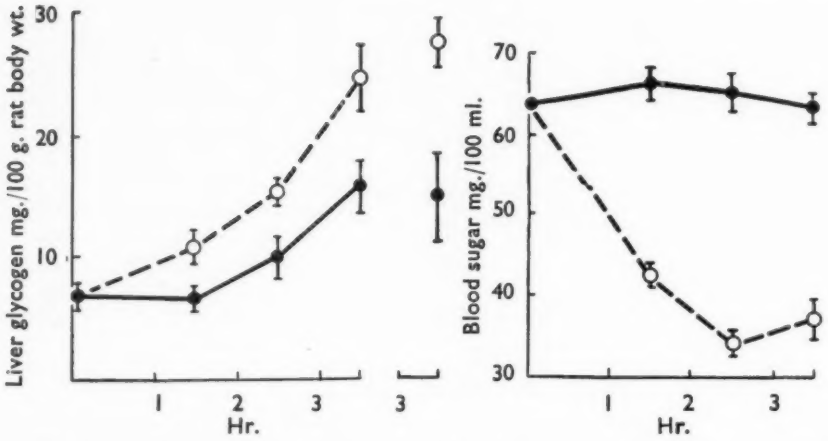

FIG. 1.—Glycogen content of livers and blood sugar levels in starved rats after treatment with oral tolbutamide (500 mg./kg.). Except where whole livers were used, the glycogen content was determined from the mean of the concentrations in portions of the right median and left lobes. Solid circles, control rats. Open circles, test rats. The points standing alone for the glycogen content at 3½ hr. were obtained from whole livers. Mean values with ± standard error are plotted.

Glycogen content was estimated using either whole livers or approximately equal portions from the right median and left lobes. When such portions were taken, it was assumed that the glycogen concentration in the liver as a whole was equal to the mean of the concentrations in the two portions examined. This assumption is supported by the evidence quoted by MacIntyre, Pedersen, and Maddock (1941) and borne out in the present experiments in which no difference was found between the concentrations in the two lobes of livers of control rats (Fig. 2). In the test animals, however, the distribution of glycogen in the two lobes was unequal, a discrepancy which is discussed below.

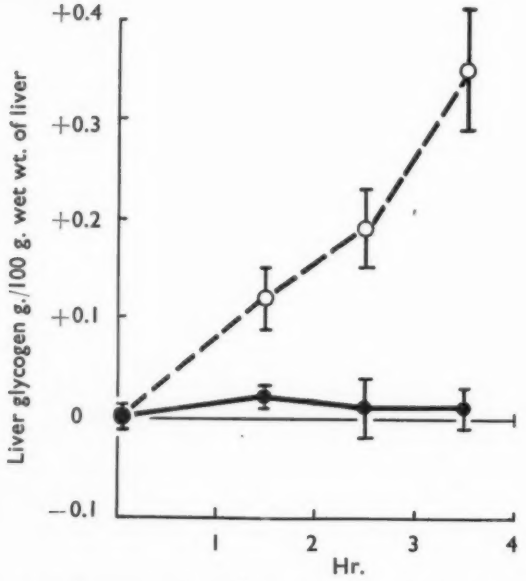

FIG. 2.—Difference in glycogen contents of liver lobes after oral tolbutamide (500 mg./kg.). Solid circles, control rats. Open circles, test rats. Mean values with ± standard errors are plotted.

Fig. 1 shows the glycogen content of the liver and the blood sugar levels at intervals after oral tolbutamide. It can be seen that in control animals given 3% sodium carbonate alone there was an appreciable rise in liver glycogen from an initial fasting level of 7.1 ± 1.0 mg./100 g. rat body weight to a value of 15.7 ± 2.3 mg./100 g. rat body weight 3½ hr. later. After oral tolbutamide, however, the rise in liver glycogen was significantly greater. Thus, when whole livers were examined, the glycogen content 3½ hr. after the drug was found to be 27.3 ± 1.9 mg. compared with 14.7 ± 3.5 mg./100 g. rat body weight in the untreated animals. Similar figures were obtained when portions of the right median and left lobes were used and the glycogen content of the whole liver calculated from the mean of the concentrations in the two portions. In test animals the value was 24.4 ± 2.8 mg./100 g. compared with 15.7 ± 2.3 mg./100 g. rat body weight in controls. Using the latter method of estimation this rise in liver glycogen was found to occur as early as 1½ hr. after oral tolbutamide. Carbutamide, when given orally, produced a comparable effect (Table III).

When portions were analysed it became apparent that the glycogen concentrations in the two lobes from treated animals were unequal. The concentration in the right median lobe was consistently higher than that in the left, the difference increasing with time to a value of 0.35 g./100 g. wet wt. of liver 3½ hr. after the administration of the drug (Fig. 2). At all times the glycogen content of the right median lobe from treated animals was significantly greater than that in the corresponding lobe from controls; the difference in the left lobes was much less marked (Fig. 3).

During the 3½ hr. period after oral tolbutamide, therefore, the liver glycogen content of fasted rats rises at a much greater rate than that of control

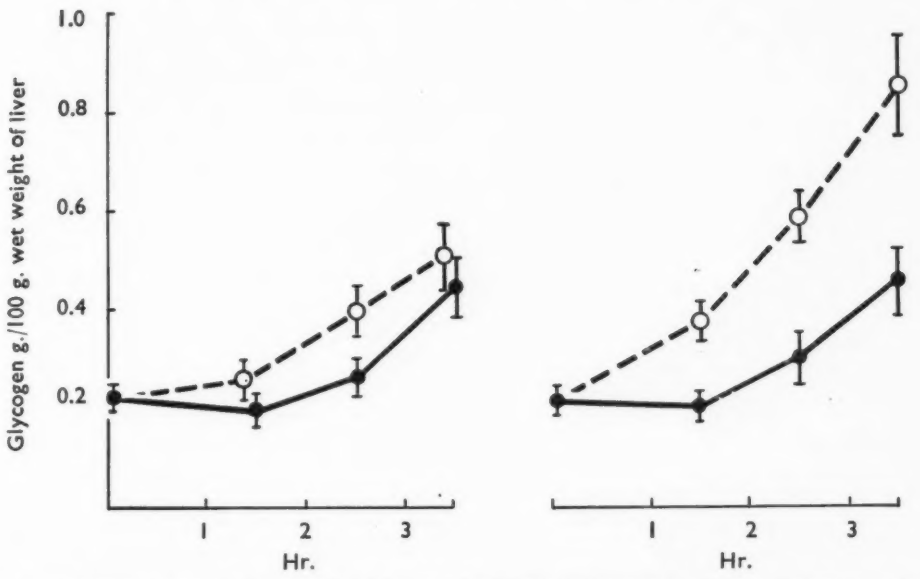

FIG. 3.—Glycogen content of liver lobes after oral tolbutamide (500 mg./kg.). The estimations from the left lobe are plotted on the left and those from the right median lobe on the right. Solid circles, controls. Open circles, test animals. Mean values with ± standard errors are plotted.

animals ; it does so asymmetrically and it does so early in the hypoglycaemic period.

It was thought possible that the asymmetrical distribution of glycogen found in the livers of treated rats might coincide with an asymmetrical distribution of the drug after its absorption from the alimentary tract due to " streamlining " in the portal circulation (Himsworth, 1950). This possibility was ruled out when it was found that the same asymmetrical distribution of glycogen occurred in animals given tolbutamide by subcutaneous injection (Table III).

Glucose-6-phosphatase.—Hawkins, Ashworth, and Haist (1956), Tybergheim *et al.* (1956), and Ashmore, Cahill, and Hastings (1956) have all demonstrated inhibition of glucose-6-phosphatase activity by carbutamide and tolbutamide *in vitro* ; Vaughan (1956), on the contrary, was unable to do so. Direct inhibition of this enzyme *in vivo*, occurring at an early stage after administration of the drug, could explain its hypoglycaemic action. To test this point glucose-6-phosphatase activity was compared in livers from normal and treated rats. Homogenates of livers from normal fasted rats (34 experiments) showed a mean glucose-6-phosphatase activity of 6.8 ± 0.2 mg. P/g. wet weight of liver/30 min.; fasted rats given carbutamide 2 hr. before killing, or tolbutamide 1 hr. or 2½ hr. before death, showed liver glucose-6-phosphatase activity well within the normal range. This finding confirms the results obtained by Ashmore *et al.* (1956). If the drugs were to inhibit glucose-6-phosphatase *in vivo* by forming an inactive enzyme-drug complex, homogenization of the livers of treated rats in 20 vol. of 1% glucose might be expected to cause dissociation of such a complex, so that the homogenates, as shown above, would exhibit normal activity. In further experiments, therefore, livers of normal and treated rats were homogenized in 1% glucose containing tolbutamide (50 mg./100 ml.) and were incubated in the presence of the drug in the same concentration. Under these conditions it was again found that the livers of normal and treated rats had the same enzyme activity. When, however, carbutamide or tolbutamide was added *in vitro* to the incubation mixture, inhibition of

TABLE III

LIVER GLYCOGEN CONTENT OF FASTED RATS 2½ HR. AFTER ORAL AND SUBCUTANEOUS SULPHONYLUREAS

Mean values ± standard error.

Drug	Route of Administration	No. of Rats	Glycogen g./100 g. Wet Weight of Liver		Total Liver Glycogen mg./100 g. Body Weight
			Left Lobe	Right Median Lobe	
Control	Oral	8	0·27 ± 0·04	0·31 ± 0·05	9·9 ± 1·6
"	Subcutaneous	6	0·22 ± 0·02	0·24 ± 0·02	6·4 ± 2·0
Tolbutamide	Oral	15	0·40 ± 0·05	0·59 ± 0·04	15·3 ± 1·2
"	Subcutaneous	14	0·37 ± 0·04	0·74 ± 0·06	19·1 ± 2·0
Carbutamide	Oral	12	0·49 ± 0·04	0·59 ± 0·05	17·0 ± 1·5

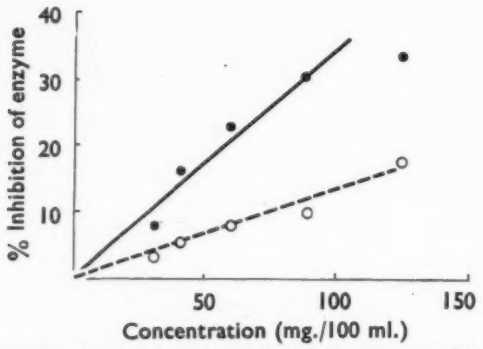

FIG. 4.—Inhibition *in vitro* of glucose-6-phosphatase by sulphonylureas. Solid circles, tolbutamide. Open circles, carbutamide. The points are mean values.

enzyme activity was detected and increased in a linear manner as the drug concentration was raised (Fig. 4). Using drug concentrations similar to those likely to occur in the blood during successful therapy (10 to 20 mg./100 ml.) very little inhibition of glucose-6-phosphatase occurred *in vitro*. Even with tolbutamide in a concentration of 125 mg./100 ml., which is close to the maximum solubility at *p*H 6.3 (Forist and Chulski, 1956), only 30 to 40% inhibition was produced.

DISCUSSION

A fall in the concentration of glucose in the blood and interstitial fluids could be due to a rise in the rate of consumption of glucose in the tissues, or to a fall in the rate of release of glucose from the liver. The results described above suggest that, as the blood sugar level falls under the influence of carbutamide or tolbutamide, there is a concomitant rise in the glycogen content of the liver, but no change in the rate of glucose consumption in the peripheral tissues. It seems unlikely, therefore, that the sulphonylureas act by stimulating the secretion of insulin, or by inhibiting its destruction. Further, the rapid rise in liver glycogen does not support the hypothesis that they act primarily by inhibiting gluconeogenesis, but suggests rather that they act by inhibiting glycogenolysis.

Glycogen is synthesized and broken down in several stages, which are shown in Fig. 5. It seems unlikely that accumulation of glycogen could result from the inhibition of any enzyme such as phosphoglucomutase, which has an action that is completely reversible. On the other hand, inhibition of any enzyme concerned solely with glycogen breakdown could produce a fall in blood sugar concentration associated with a rise in liver glycogen content; glucose-6-phosphatase and the "debranching" enzyme fall into this category. It should also be mentioned that although liver phosphorylase catalyses a reversible reaction, Cahill, Ashmore, Zottu, and Hastings (1957) have recently reported experiments which suggest that the predominant direction of this reaction can be influenced *in vitro* by sodium and potassium ions and by glucagon and adrenaline. Even under ionic conditions which favour glycogenolysis least (high potassium and low sodium concentrations), glucagon *in vitro* has a marked glycogenolytic effect. It is, therefore, possible that inhibition of phosphorylase itself could lead to inhibition of glycogenolysis.

Accumulation of glycogen in the livers of patients suffering from one form of glycogen storage disease is accompanied by a reduction in glucose-6-phosphatase activity of the liver, hypoglycaemia and an impaired response to the hyperglycaemic action of exogenous adrenaline and glucagon (Andersen, 1952).

The results outlined above suggest that the accumulation of liver glycogen found in rats treated with tolbutamide is not due to glucose-6-phosphatase inhibition. In this respect they support the clinical findings of Moorhouse and Kark (1956), Fajans, Louis, Seltzer, Johnson, Gittler, Hennes, Wajchenberg, Ackerman and Conn (1956) and Butterfield, Camp, Hardwick and Holling (1957), who, among others, have shown that the response of treated patients to exogenous adrenaline and glucagon is normal.

Although it seems unlikely that these drugs act by inhibiting glucose-6-phosphatase, there remains the possibility that they might inhibit the breakdown of glycogen to glucose-1-phosphate either directly or indirectly. The action of the sulphonylureas upon the release of glucose from liver slices

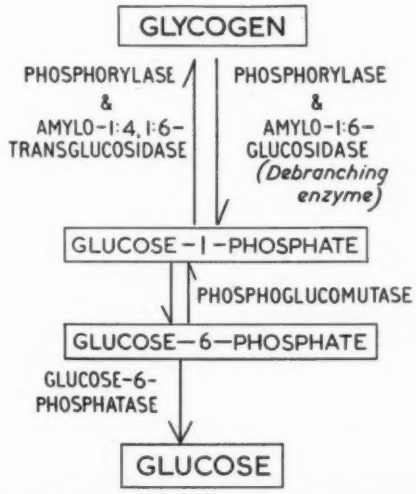

FIG. 5.—Mechanism of glycogenolysis in the liver.

has been studied by several workers (Clarke *et al.*, 1956; Berthet, Sutherland, and Makman, 1956; Vaughan, 1956; Mohnike and Knitsch, 1956). The results of these experiments are conflicting, but do not suggest that the drugs act by inhibiting the phosphorylase mechanisms directly. They could, however, act indirectly by inhibiting the release of hormones or other substances which stimulate this reaction, such as adrenaline and glucagon, both of which are known to stimulate it *in vivo* and *in vitro*.

Houssay and Penhos (1956) and Lang and Sherry (1956) showed that adrenalectomized animals are exceedingly sensitive to tolbutamide, and that hypoglycaemia develops even if cortical hormones are given concurrently. Grant (personal communication) has shown that in a rabbit with a denervated ear, hypoglycaemia produced by intravenous tolbutamide is accompanied by release of endogenous adrenaline, which produces a normal hyperglycaemic effect. In the adrenalectomized rabbit no such adrenaline response occurs after the drug and the blood sugar falls until convulsions develop, yet exogenous adrenaline in such a rabbit exerts its normal hyperglycaemic effect. This evidence indicates that sulphonylureas do not interfere to any appreciable extent with the release or action of adrenaline *in vivo*, a conclusion which is supported by clinical findings.

It is not possible, on present evidence, to explain the accumulation of liver glycogen which occurs soon after the sulphonylureas are administered to rats. Bertram, Bendfeldt, and Otto (1955) and Anderson, Perfetto, Termine, and Monaco (1956) have suggested that these drugs might interfere with the release of glucagon from the pancreas; they are not likely to interfere with the action of glucagon for the reasons already given. Glucagon, according to Cahill *et al.* (1957), exerts a glycogenolytic action upon the phosphorylase reaction, so that a reduction in the amount of glucagon reaching the liver could result in a decrease in the rate of glycogenolysis and hence an accumulation of liver glycogen. The asymmetrical distribution of this accumulated glycogen cannot be explained and it is not apparently due to uneven distribution of the drug in the liver following absorption from the alimentary tract; oral and parenteral tolbutamide produce the same effect.

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References

Andersen, D. H. (1952). *Carbohydrate Metabolism*, p. 28. Baltimore: The Johns Hopkins Press.

Anderson, G. E., Perfetto, A. J., Termine, C. M., and Monaco, R. R. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 340.

Ashmore, J., Cahill, G. F., and Hastings, A. B. (1956). *Metaboli m*, **5**, 774.

Bander, A., and Scholz, J. (1956). *Dtsch. med. Wschr.*, **81**, 889.

Beringer, A., and Lindner, A. (1956). *Wien. klin. Wschr.*, **68**, 316.

Berthet, J., Sutherland, E. W., and Makman, M. H. (1956). *Metabolism*, **5**, 768.

Bertram, F., Bendfeldt, E., and Otto, H. (1955). *Dtsch. med. Wschr.*, **80**, 1455.

Butterfield, W. H. J., Camp, J. L., Hardwick, C., and Holling, H. E. (1957). *Lancet*, **1**, 753.

Cahill, G. F., Ashmore, J., Zottu, S., and Hastings, A. B. (1957). *J. biol. Chem.*, **224**, 237.

Clarke, D. W., Davidson, M., Schonbaum, E., and Serman, H. (1956). *Canad. med. Ass. J.*, **74**, 966.

Ernster, L., Zetterström, R., and Lindberg, O. (1950). *Acta chem. scand.*, **4**, 942.

Fajans, S. S., Louis, L. H., Seltzer, H. S., Johnson, R. D., Gittler, R. D., Hennes, A. R., Wajchenberg, B. L., Ackerman, I. P., and Conn, J. W. (1956). *Metabolism*, **5**, 820.

Forist, A. A., and Chulski, T. (1956). Ibid., **5**, 807.

Gey, G. O., and Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.

Good, C. A., Kramer, H., and Somogyi, M. (1933). *J. biol. Chem.*, **100**, 485.

Hawkins, R. D., Ashworth, M. A., and Haist, R. E. (1956). *Canad. med. Ass. J.*, **74**, 972.

Himsworth, H. P. (1950). *The Liver and its Diseases*, 2nd ed., p. 27. Oxford: Blackwell.

Houssay, B. A., and Penhos, J. C. (1956). *Metabolism*, **5**, 727.

King, E. J. (1951). *Micro-analysis in Medical Biochemistry*, 2nd ed., p. 20. London: Churchill.

Lang, S., and Sherry, S. (1956). *Metabolism*, **5**, 733.

Levine, R., and Duncan, G. G. (1956). Ibid., **5**, 721.

MacIntyre, D. S., Pedersen, S., and Maddock, W. G. (1941). *Surgery*, **10**, 716.

Miller, W. L., and Dulin, W. E. (1956). *Science*, **123**, 584.

Mohnike, G., and Knitsch, W. (1956). *Dtsch. med. Wschr.*, **81**, 891.

Moorhouse, J. A., and Kark, R. M. (1956). *Metabolism*, **5**, 847.

Strickland, K. P., Thompson, R. H. S., and Webster, G. R. (1956). *J. Neurol. Psychiat.*, **19**, 12.

Swanson, M. A. (1950). *J. biol. Chem.*, **184**, 647.

Tybergheim, J. M., Halsey, Y. D., and Williams, R. H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 322.

Vallance-Owen, J., and Hurlock, B. (1954). *Lancet*, **1**, 68.

Vaughan, M. (1956). *Science*, **123**, 885.

Brit. J. Pharmacol. (1957), **12**, 356.

LIPID BIOSYNTHESIS AND CHEMICALLY INDUCED PARALYSIS IN THE CHICKEN

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Dyflos (di*iso*propylphosphorofluoridate, DFP) retards the synthesis of lipid in the sciatic nerve of the chicken *in vivo*. A similar inhibition occurs *in vitro*, but only at relatively high concentrations (10^{-3}M) of the drug. The major portion of the ^{14}C supplied as acetate, and incorporated into lipid, is found with the fatty acids. Attempts to separate the lipids involved by paper chromatography were unsuccessful.

The anticholinesterase properties of substances which are capable of producing "ginger" paralysis in man and experimental animals have led some workers to the belief that a direct correlation exists between these two properties. Bloch (1941) and Hottinger and Bloch (1943) considered that the neurotoxic syndromes resulting from triorthocresol phosphate (TOCP) were due to inhibition of true-cholinesterase at motor end-plates. It was subsequently shown, however, that TOCP is a highly specific inhibitor of pseudo-cholinesterase, an enzyme which does not occur at motor end-plates (Mendel and Rudney, 1944; Earl and Thompson, 1952a).

Earl and Thompson (1952b) followed cholinesterase levels in the nervous system of chickens poisoned with TOCP. They concluded that the biochemical lesion which led ultimately to paralysis was the prolonged depression of pseudo-cholinesterase activity, particularly in the spinal cord. As supporting evidence they pointed out that all known compounds which produce "ginger" paralysis are selective inhibitors of pseudo-cholinesterase. Davison (1953) and Austin and Davies (1953) examined a wide range of anticholinesterases, many of them highly selective inhibitors of the pseudo-enzyme. Further, Austin and Davies (1953) maintained the pseudo-cholinesterase activity of chicken spinal cord at a low level for 21 days with methyl*iso*propylphosphonofluoridate, a non-paralysing anticholinesterase, but this treatment did not lead to paralysis.

The biosynthesis of phospholipid in the nervous system of the chicken has been shown by Webster (1954) to be unaffected by TOCP. Strickland, Thompson, and Webster (1956) found that dyflos (di*iso*propylphosphorofluoridate (DFP) and NN'-di*iso*propylphosphorodiamidic fluoride (mipafox) inhibit oxygen uptake and oxidative phosphorylation in chicken brain slices only at very high concentrations of the drug, and they concluded that these effects were unlikely to play a part in degeneration of the nerve fibres.

The insecticide mipafox has been shown by Barnes and Denz (1953) to cause paralysis in chickens. The repeated injection of mipafox into rats results in an abnormal metabolism of the sciatic nerves (Majno and Karnovsky, 1955). When these nerves were incubated with ^{14}C acetate the rate of oxygen consumption was depressed 16% and of ^{14}C incorporation into lipid 50%.

Majno and Karnovsky (1955) confined their studies to animals which had been exposed to mipafox for a period of three to five weeks. Although rats are not susceptible to nerve degeneration produced in this way, the period of time between the initial administration of the drug and the examination of the nerve tissue is such that changes in metabolism found could be of a secondary nature and not necessarily directly connected with the primary biochemical lesion.

A similar study has been made here of some aspects of the metabolism of sciatic nerve of the fowl, when poisoned with dyflos, and results similar to those of Majno and Karnovsky obtained.

METHODS

The Formation of Nerve Lipids.—Both sciatic nerves were dissected from normal or dyflos-treated birds as quickly as possible after death and adhering

tissues and fat removed by stroking with a scalpel blade. The nerves were quickly weighed and placed in an ice-cold solution of phosphate buffer (Krebs and Henseleit, 1932) until used. After drying with filter paper, the nerves were placed in Warburg vessels.

When the effect of dyflos was being studied *in vivo*, 1.5 ml. of Krebs–Henseleit phosphate containing ^{14}C acetate was added to the flask. For *in vitro* studies, 1.0 ml. of the buffer containing ^{14}C acetate was added, after the addition of 0.5 ml. of buffer containing dyflos. To all flasks was added 0.3 ml. of a solution containing 60 μM./ml. of glucose and 24 μM./ml. of sodium acetate. The centre well contained rolls of No. 40 Whatman paper saturated with 0.2 ml. of 30% potassium hydroxide, and the side arm 0.3 ml. of 5 N sulphuric acid. The vessels were gassed with oxygen and incubated for 3 hr. at 37°.

Readings of oxygen consumption were taken at 30 min. intervals, and the uptake was linear over 3 hr. Enzyme action was stopped by tipping the acid from the side arm. The flasks were left overnight in the cold room, after which the nerves were transferred to stoppered test tubes, and washed 20 times with 2 ml. portions of 0.01 M sodium acetate. They were finally washed with 0.01 N hydrochloric acid and dried in a desiccator over sodium hydroxide and calcium chloride, or alternatively they were freeze dried. The final acid washing and subsequent drying was performed to ensure removal of all acetate.

When dry, the nerves were homogenized in a Potter–Elvehjem homogenizer (Potter, 1945) in a 2:1 chloroform:methanol mixture. Water, equal to one-fifth of the volume of solvent, was added, and the tube contents thoroughly mixed (Folch, Lees, and Sloane-Stanley, 1954).

The chloroform layer was centrifuged down and transferred to a clean vessel. The solution was made up to a volume of 10 ml. from which 2 ml. aliquots were removed to an oxidizing vessel (Calvin, Heidelberger, Reid, Tolbert, and Yankwi[illegible] 1949).

After removal of the solvent on the water bath, the lipid extracts were oxidized to carbon dioxide (Calvin *et al.*, 1949), which was collected in a saturated solution of barium hydroxide. The barium carbonate was washed twice in the centrifuge with carbon-dioxide-free water and once with acetone. It was suspended in ethanol and transferred with a Pasteur pipette to a weighed 1 in. diameter stainless steel planchette and dried slowly under an infra-red lamp. When dry it was weighed and counted in a Tracerlab gas flow Geiger counter. All counts were corrected to infinite thinness in a sample area of 4.7 sq. cm. by multiplying by the appropriate factor determined from the sample weight. The filter paper rolls were removed from the Warburg flasks and placed in a 25 ml. standard flask. The centre well of the Warburg vessel was washed out six times with small quantities of carbon-dioxide-free distilled water and transferred to the volumetric flask.

The flasks were made up to volume and allowed to stand overnight. 5 ml. aliquots were placed in a centrifuge tube and to this was added 0.5 ml. of 0.29 M potassium carbonate as carrier, and 2 ml. of 0.2 M barium chloride. The precipitate barium carbonate was washed, plated, and counted as before.

The Separation of Lipids.—An attempt was made to separate the extracted lipids by a modification of the spot test method of Hack (1953), who used filter paper disks. Strips of Whatman No. 3 paper, 28 cm. by 7.5 cm., were washed with a 4:1 chloroform:methanol mixture and dried. 1 ml. of the lipid extract containing approximately 2 mg. of lipid was applied slowly, while being dried with a current of hot air, to a line 4 cm. from the bottom of the filter paper strip. The paper was suspended in a jar containing 4:1 chloroform:methanol for half an hour. Filter paper strips, saturated with solvent, lined the walls of the jar to maintain a saturated solvent vapour within the jar. After equilibration the lower end of the paper was immersed in the solvent and the solvent front allowed to run to a pencil line 5 cm. from the origin. The paper was dried and the process repeated twice. Acetone was run in a similar manner to a line 20 cm. from the origin. Two more runs were carried out with acetone, the paper being dried between each run. Finally four runs were made with [illegible]anol to a line 15 cm. from the origin.

The initial papers were cut into strips longitudinally and the following tests applied: (1) The paper was exposed to acid ammonium molybdate, followed by H_2S vapour (Hanes and Isherwood, 1949). A blue band showed up phosphorus against a buff background. (2) 0.01% ninhydrin in butanol was sprayed on the paper which was subsequently dried at 105°. Amine lipids produced a typical purple ninhydrin colour. (3) The paper was dipped in a cooled 1:1 sulphuric acid:acetic anhydride mixture. A pink to blue stain showed the presence of cholesterol. (4) Exposure to osmic acid vapour produced a brown to black band at the site of unsaturated fatty acids. Staining with fat stains such as Nile blue and Sudan IV was too unspecific to be of any value. Hack (1953) stated that the exposure of choline lipids to iodine shows the presence of choline as a transient yellow-brown colour. While this has been found to be so, Brante (1949) has shown that the test is not specific for choline.

For ^{14}C measurement the papers were cut into strips transversely corresponding to the positions of the lipids as shown by the spot tests. These strips were cut up finely with scissors and extracted four times with 4:1 chloroform:methanol. The extracts were filtered into Van Slyke oxidizing vessels where the solvent was evaporated. Lipids extracted from paper corresponding to the acetone front were saponified with 5 ml. of 10% KOH in 50% ethanol at 100° overnight. The ethanol was removed by evaporation and non-saponifiable lipids removed by extraction with four portions of petroleum ether (60 to 80°), each of 4 ml. The aqueous layer was extracted, after acidification, with six 4 ml. lots of ether. The extracted lipids were then oxidized and

the resulting barium carbonate washed, plated, and counted.

The Isolation of Fatty Acids.—The distribution of isotopic carbon was studied in another way, namely by a separation of the lipids into non-saponifiable matter, fatty acids, and fat alcohols. The saponification technique of Masoro, Chernick, Chaikoff, and Felts (1950) was applied to the lipid extracts. The petroleum ether extracts of non-saponifiable matter and the ether extracts of fatty acids were dried on the water bath, while the aqueous solution remaining after precipitation of fatty acids was freeze dried. All three samples were oxidized and counted as before.

Results

Majno and Karnovsky (1955) confined their studies to the metabolic state of nerves after prolonged exposure to mipafox. It is important, however, to learn what changes occur during the latent period which, in the fowl, extends up to fourteen days after dyflos ingestion. Groups of birds were injected subcutaneously with dyflos 1 mg./kg. and atropine, and, from these, individuals were killed at intervals. At the same time a comparable number of normal animals were killed as controls. The sciatic nerves were removed and the rate of oxygen consumption, carbon dioxide production and lipid synthesis measured. Table I summarizes the results.

TABLE I
THE METABOLISM OF FOWL PERIPHERAL NERVE AFTER DYFLOS INTOXICATION *IN VIVO*

Time of Killing (Days)	No. of Nerves/ Group	O_2 Consumption/ 100 mg. of Nerve μl. O_2/hr.		$^{14}CO_2$ Production/ 100 mg. of Nerve Counts/min.		Lipid Synthesis/ 100 mg. of $BaCO_3$ Counts/min.	
		Control	Dyflos	Control	Dyflos	Control	Dyflos
1	13	12·24 ±0·65	11·48 ±0·43	13,520 ±960	13,420 ±1,040	444 ±74	266 ±28
5	8	7·1 ±0·25	8·4 ±0·42	7,766 ±515	8,560 ±802	341 ±27	319 ±21
17	5	7·7 ±0·69	7·7 ±0·84	3,789 ±129	4,140 ±468	175 ±21·6	134 ±22·5

Oxygen consumption is expressed as μl./100 mg. of fresh nerve/hr., and carbon dioxide production as counts/100 mg. of barium carbonate/min. Since Folch *et al.* (1954) have found that the chloroform layer of the lipid extract contains all lipids, and that no other material is extracted, lipid isotope content has been referred to the mass of barium carbonate formed from the oxidation of the lipid, rather than to dry weight of lipid. The rate of lipid synthesis then is expressed as counts/min./100 mg. of barium carbonate.

Twenty-four hours after the administration of dyflos there is a significant fall in the rate of formation of lipid from acetate. At 5 and 17 days the rate is still lower in the experimental group than in the control group.

The action of dyflos on lipid biosynthesis by sciatic nerve is shown in Table II.

TABLE II
THE METABOLISM OF FOWL PERIPHERAL NERVE AFTER DYFLOS INTOXICATION *IN VITRO*

Dyflos Conc. M	No. of Nerves/ Group	O_2 Consumption/ 100 mg. of Nerve μl. O_2/hr.		$^{14}CO_2$ Production/ 100 mg. of Nerve Counts/min.		Lipid Synthesis/ 100 mg. of $BaCO_3$ Counts/min.	
		Control	Dyflos	Control	Dyflos	Control	Dyflos
10^{-2}	2	11·28	5·68	15,500	3,135	1,182	228
10^{-3}	11	7·84 ±0·53	7·31 ±0·52	8,935 ±1,162	7,950 ±1,064	349 ±22·4	230 ±21·2
10^{-4}	5	7·90 ±0·72	10·00 ±0·56	7,905 ±1,800	8,800 ±774	422 ±84	360 ±118

A concentration of 10^{-2} M dyflos reduces very considerably the rate of lipid synthesis, but at the same time markedly inhibits the rate of oxygen consumption and of carbon dioxide production of the nerve tissue.

At a concentration of 10^{-3} M, however, when t is calculated on the basis of a paired experiment, the difference in the rates of lipid synthesis between control and experimental groups is significant at the 0.1% level. No difference was found between the rates of oxygen uptake or carbon dioxide output in the control or experiment groups, when submitted to a t test. At 10^{-4} M the trend towards reduction of lipid synthesis is still apparent, although no longer statistically significant.

The extent of chromatographic separation of lipids may be seen in Table III. A complete

TABLE III
THE REACTION OF LIPIDS AT SOLVENT FRONTS TO VARIOUS SPOT TESTS
The probable lipids present shown in the last column are based on the known solubilities of the lipids indicated.

Solvent Front	Phosphate	Ninhydrin	Cholesterol	Osmic Acid	Probable Lipids Present
Origin	Trace	Small	Nil	Small	
$CHCl_3+CH_3OH$ . . .	Intense	Intense	,,	Intense	Sphingomyelin, phosphatidyl-serine, diphosphoinositide gangliosides, sulphatides
CH_3OH	Medium	,,	,,	Medium	Lecithin, plasmalogens, phosphatidyl-ethanolamine
Acetone	Trace	Nil	Intense	Intense	Cholesterol, neutral fat, free fatty acids

separation was not possible by this technique, but a useful differentiation into lipid classes was obtained.

The recovery of isotopic carbon after the application of this technique was poor, however, only 37 to 54% recovery being obtained (Table IV).

TABLE IV
% RECOVERY OF ISOTOPIC CARBON IN LIPIDS SEPARATED BY VARIOUS SOLVENTS ON PAPER

Origin	$CHCl_3$+ CH_3OH	CH_3OH	Acetone		Total Recovery
			Non-sapon.	Fatty Acids	
0	8·2	10·3	7·5	12·3	38·3
0	8·7	6·3	9·3	12·9	37·2
0	13·4	5·0	11·6	24·6	54·6
0	10·2	6·6	15·7	13·3	45·8
0	11·3	8·9	11·3	16·0	47·2

About half of the lipid, in terms of ^{14}C content, was found to be firmly bound to the paper. This was demonstrated in two ways. A control sample of 0.4 ml. of the original chloroform: methanol extract of nerve was transferred to an oxidizing vessel and the lipid solute oxidized. First 0.4 ml. of extract was allowed to spread on Whatman No. 3 paper and, immediately after drying, the lipid was extracted from the paper with chloroform: methanol. Secondly 0.4 ml. was dried on to a line on a filter paper strip as before and chromatogrammed with chloroform: methanol. The lipid which travelled with the solvent front was extracted with chloroform: methanol. Of the added ^{14}C, 45.4% was recovered from the unchromatogrammed sample and 50.5% from the chromatogrammed lipid. A further 5.6% of the added isotope was recovered with hydrochloric acid from the unchromatogrammed sample, but neither acidic nor basic solvents were capable of extracting more isotope (Table V).

TABLE V
% RECOVERY OF ISOTOPIC CARBON IN LIPIDS ABSORBED ON VARIOUS REGIONS OF FILTER PAPER
The lipid stain on paper was not chromatogrammed.

Eluent	Lipid Stain on Paper	$CHCl_3+CH_3OH$ Front	Origin
$CHCl_3+CH_3OH$ (4: 1)	45·4	50·5	—
1 N HCl in $CH_3OH+C_2H_5OH$ (1: 1)	5·6	0	0
Pyridine	0	—	0
Total recovery ..	51	50·5	—

When the lipids were saponified, and fatty acids obtained by soxhlet extraction of the filtered precipitate resulting from acidification of saponified material, very poor recovery of the ^{14}C was obtained. Extraction of the precipitated fatty acids with ether in a separating funnel, however, resulted in a correct balance sheet, as is shown in Table VI. This suggests that a considerable amount of the label is being lost during the Masoro procedure probably in the form of low molecular weight fatty acids.

TABLE VI
TYPICAL RECOVERIES OF ^{14}C IN FRACTIONS OBTAINED FROM SAPONIFIED LIPID, EXPRESSED AS COUNTS/MIN. CALCULATED TO INFINITE THINNESS

	Total	Non-saponifiable	Fatty Acids	Alcohols	Sum of Fractions
Fatty acids soxhlet extracted ..	550	38	208	25	271
Fatty acids extracted in separating funnel ..	262	42	216	—	258

DISCUSSION

In this type of chronic poisoning, the observed symptoms are only secondary. In the case of dyflos poisoning, very little free dyflos is present in tissues, even 20 min. after its administration, whereas the first signs of chronic poisoning take from 8 to 14 days to appear. The biochemical change which finally produces nerve degeneration probably occurs then shortly after dyflos absorption. Smith and Lillie (1931) thought that the delay in the onset of symptoms produced by TOCP intoxication was due to a slow release of the compound from lipoidal tissue in which it was dissolved, but this now appears to be unlikely.

The lipid components of nerve amount to more than 50% of the dry weight of that tissue, and consequently any interference with the maintenance of this lipid fraction could profoundly affect the overall function as well as structure of the nerve. The ingestion of dyflos by fowls leads to a significant fall in the rate of synthesis of lipid during the first day of poisoning. There is still a trend towards a lower rate of synthesis at 5 and at 17 days. Since the fall is most marked shortly after dyflos absorption, and the effect is produced on such an important component of nerve, namely lipid, it may well be that this depression of lipid synthesis is the initial cause of the degenerative changes. Although a recovery of the synthetic mechanism is seen, the nerve may not be able to repair the damage sufficiently to prevent the ultimate result of complete degeneration of nerve structure.

In vitro, dyflos inhibits lipid synthesis. This inhibition is most marked at a dyflos concentration of 10^{-2} M, but there is also a very considerable depression of respiration by the nerve, and hence no conclusion can be reached regarding the action

of dyflos, at this concentration, on lipid synthesis. When lower dyflos concentrations are used, however, there is still a marked depression of the rate of lipid synthesis. The difference between means at 10^{-3} M dyflos, as shown in Table II, is significant at the 0.1% level when subjected to a *t* test, while no difference exists between the means of the controls or the experimental groups in the columns showing the rates of oxygen consumption and carbon dioxide production.

When the concentration of dyflos is reduced to 10^{-4} M an inhibition is still evident, although small. These results support those obtained from the series of experiments *in vivo*.

Although organo-phosphorus anticholinesterase drugs are commonly considered as substances which inhibit cholinesterase at extremely low concentrations *in vitro*, and thus exert their toxic action *in vivo*, this high affinity for enzymes need not always be necessary for an effect to be produced *in vivo*. A discrepancy exists between *in vivo* and *in vitro* results which cannot be adequately explained at present. It is perhaps significant in this regard that a relatively high dosage of dyflos must be administered to the animals to produce the neuritis. The dosage required for fowls is so high that death would result in the majority of the animals if atropine were not given together with the dyflos. If an even distribution of dyflos throughout the fowl tissues is assumed, the concentration would be no higher than 5.5×10^{-6} M. This concentration is too low to produce any substantial effect on lipid synthesis *in vitro*, but *in vivo* a 1 mg./kg. dose results in a significant inhibition.

Separation of the extracted lipid by paper chromatography was unsuccessful. The results obtained suggest that about half of the labelled lipid is firmly bound to filter paper. The absorbed material may be largely kephalin, since Hack (1953) states that kephalin is strongly absorbed on filter paper, and he suggests that all extracts should be filtered through sintered glass, for this reason.

The incomplete recovery of ^{14}C in the fractions separated after saponification is probably due to loss of ^{14}C incorporated into fatty acids of low molecular weight. Such fatty acids would remain in solution after the acidification of the saponified material, and would subsequently be removed during the freeze-drying operation. Table VI shows that at least 80% of the total counts are due to ^{14}C in fatty acids. About 10% of the label appears in the non-saponifiable fraction and less than 5% with the alcohols. In view of this, dyflos must depress lipid synthesis during the formation of fatty acids.

It is perhaps significant that a similar effect can be obtained with fowls, after one dose of a paralysing agent, to that which results when rats are subjected to repeated doses of such an agent. Rats, which are not susceptible to this form of paralysis, require prolonged treatment with the drug to produce any degenerative changes in nerve tissue.

I am grateful to Professor V. M. Trikojus for his advice and interest during the course of this work. I am also grateful to the Chief Scientist, Department of Supply, for permission to undertake this work.

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References

Austin, L., and Davies, D. R. (1954). *Brit. J. Pharmacol.*, **9**, 145.
Barnes, J. M., and Denz, F. A. (1953). *J. Path. Bact.*, **65**, 597.
Bloch, H. (1941). *Helv. med. acta*, **8**, Suppl. 7, 15.
Brante, G. (1949). *Nature, Lond.*, **163**, 651.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E. (1949). *Isotopic Carbon*, p. 92. New York: Wiley.
Davison, A. N. (1953). *Brit. J. Pharmacol.*, **8**, 212.
Earl, C. J., and Thompson, R. H. S. (1952a). Ibid., **7**, 261.
—— —— (1952b). Ibid., **7**, 685.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1954). *Fed. Proc.*, **13**, 209.
Hack, M. H. (1953). *Biochem. J.*, **54**, 602.
Hanes, C. S., and Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hottinger, A., and Bloch, H. (1943). *Helv. chim. acta*, **26**, 142.
Krebs, H. A., and Henseleit, K. (1932). *Hoppe-Seyl. Z.*, **210**, 33.
Majno, G., and Karnovsky, M. L. (1955). *Fed. Proc.*, **14**, 250.
Masoro, E. J., Chernick, S. S., Chaikoff, I. C., and Felts, J. M. (1950). *J. biol. Chem.*, **185**, 845.
Mendel, B., and Rudney, H. (1944). *Science*, **100**, 499.
Potter, V. R. (1945). *Manometric Techniques and Tissue Metabolism*, ed. by Umbreit, W. W., Burris, R. H., and Stauffer, J. F., p. 136. Minneapolis: Burgess.
Smith, M. I., and Lillie, R. D. (1931). *Arch. Neurol. Psychiat., Chicago*, **26**, 976.
Strickland, K. P., Thompson, R. H. S., and Webster, G. R. (1956). *Biochem. J.*, **62**, 512.
Webster, G. R. (1954). Ibid., **57**, 153.

Brit. J. Pharmacol. (1957), **12**, 361.

THE PRESERVATION OF SUBSTANCE P BY LYSERGIC ACID DIETHYLAMIDE

BY

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(RECEIVED APRIL 25, 1957)

Lysergic acid diethylamide (LSD) potentiated the response of guinea-pig ileum to substance P but not to histamine. It also inhibited the disappearance of substance P when incubated with guinea-pig brain extract but not when incubated with chymotrypsin. Eserine, morphine, mescaline, chlorpromazine, ergometrine, strychnine and 2 bromo-LSD did not have this effect. Oxytocin was not destroyed by brain extract. The inhibition of the destruction of substance P by LSD could be antagonized by 2 bromo-LSD. This effect of LSD may have some relation to its pharmacological actions.

This paper concerns an evaluation of lysergic acid diethylamide (LSD) and various other drugs for their ability to inhibit the enzymatic destruction of substance P *in vitro*. The study was initiated when, during the course of an investigation of the contractile response of plain muscle to substance P, it was observed that LSD, in concentrations below 10^{-6} (Fig. 1), potentiated the action of this polypeptide on guinea-pig ileum, whereas the action of histamine was not potentiated. Since Gullbring (1943) described an enzyme capable of destroying substance P at a fairly rapid rate, it seemed possible that the potentiation was due to the preservation of substance P from enzymatic destruction.

METHODS

Acetone-dried powders of guinea-pig brain were used as a source of the enzyme. The extract was found to be thermolabile, and consequently the powder was stored in a deep freeze until ready for use, at which time an aliquot was ground with Tyrode (1 to 2 mg./ml.) and centrifuged for 2 hr. at 4° and 2,000 rev./min. The optimal *p*H for activity of this enzyme was found to lie between 6 and 7 by the method described below. This fact suggested that this system was similar to that described by Gullbring.

The substance P used in these experiments was prepared by Dr. T. B. B. Crawford of this laboratory. It was extracted from horse intestine by the method described by Amin, Crawford and Gaddum (1954), and contained 13 units/mg. Part of this was further purified by the method of Pernow (1953), and contained 60 units/mg. Experiments were conducted with both of these preparations.

To test the potency of the brain extract and the modifications of its activity by pharmacological agents, the following mixture was prepared and incubated at 37°. Substance P (5 units/ml. in Tyrode) 0.5 ml.; 0.1 ml. of the brain extract supernatant; 0.1 ml. of the drug or drugs to be tested for inhibition of enzymatic activity, and Tyrode solution to make up 1.0 ml. In order to slow the reaction to a rate which could be measured accurately and to mitigate the disappearance of the LSD in the brain extract (Rothlin, 1956), the quantities of the brain extract were less than those used by Gullbring. After incubation the sample was cooled and an aliquot assayed on guinea-pig ileum in terms of a substance P standard in the presence of atropine 10^{-7}. Except where noted, drugs were applied to the guinea-pig ileum for bioassay purposes for a period of 30 sec. In certain experiments chymotrypsin (0.1 ml. of 5×10^{-6}) was used in place of the brain extract. The composition of the incubation mixture was kept constant in all other respects.

The guinea-pigs used as a source of enzyme and for bioassay purposes weighed between 150 and 200 g.

LSD and 2-bromo-lysergic acid diethylamide (BrLSD) were kindly presented by Sandoz Products. Crystalline chymotrypsin was obtained from the Armour Laboratories.

RESULTS

The LSD potentiation of substance P was determined on the guinea-pig ileum. In these experiments the preparation was exposed to standard concentrations of substance P for periods of 50 sec. In some experiments alternate doses of histamine and substance P were applied. After obtaining a constant response, LSD was introduced into the bath 1 to 2 min. prior to the histamine or substance P. Fig. 1 illustrates a typical result: the response of the guinea-pig ileum to substance P was potentiated, whereas that to histamine was

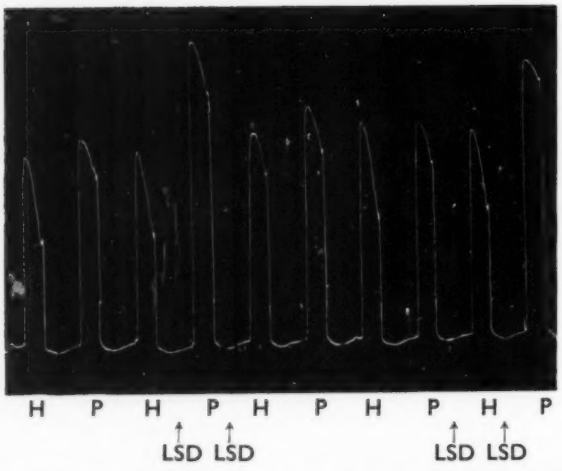

FIG. 1.—Guinea-pig ileum. 3 ml. bath. Potentiation of substance P but not of histamine by LSD. H=histamine, 15 ng.; P=substance P, 0.25 units. At the arrows, 0.1 ml. LSD 10^{-6} was introduced into the bath 1.5 min. before H or P.

not. The potentiation appeared within 1.5 min. and was reversible. If the duration of exposure to substance P was decreased to 30 sec. in these experiments, potentiation was not observed.

Fig. 2 shows that when substance P was incubated with the brain extract it was progressively inactivated, whereas substance P incubated with LSD and the brain extract was spared. The brain extract, with or without LSD, did not interfere with assays for substance P. Nearly full activity was found in the presence of fresh brain extract (zero time) or in the presence of extract that had been incubated for 2 hr. (encircled points).

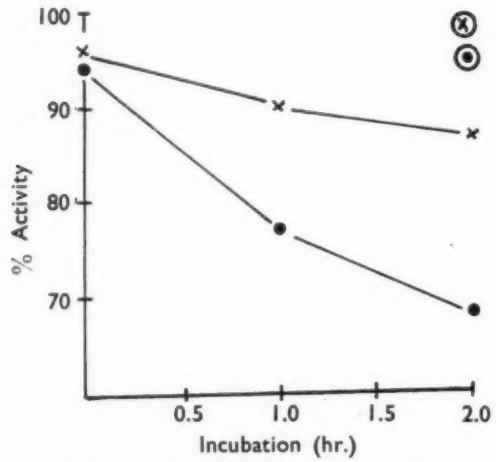

FIG. 2.—The % activity remaining after incubating substance P for various periods with brain extract without LSD (●) and in the presence of LSD 10^{-8} (X). The points enclosed in circles show the results obtained in control experiments when substance P was added after incubation.

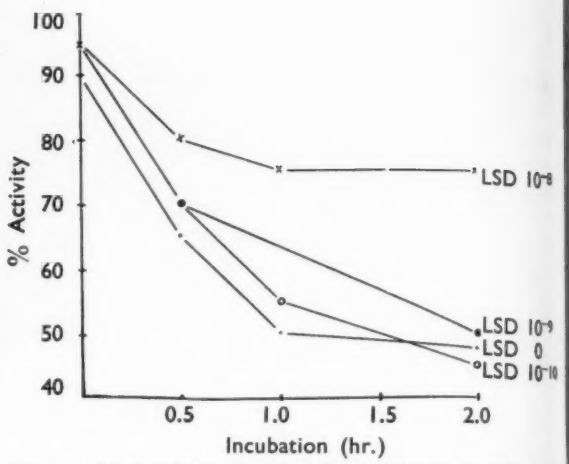

FIG. 3.—The % activity remaining after incubation of substance P with brain extract in the presence of various concentrations of LSD.

Fig. 3 illustrates the effects of various doses of LSD on the speed of inactivation of substance P. The ED50 appears to lie between 10^{-8} and 10^{-9}. It should be noted that although the destruction of substance P appeared to cease in this experiment it was more frequently found that, given a proper relationship between substrate and enzyme, the substance P activity was continuously reduced as long as it was followed.

It is important to note that, if either the period of incubation was increased or too much brain extract was used in the incubation mixture, the two curves representing the mixtures with and without LSD tended to converge. In the latter case, this could proceed at a very rapid rate, so that after the first few minutes of incubation no difference appeared between the variously treated incubation mixtures. This phenomenon is probably due to the disappearance of LSD in the brain extract (Rothlin, 1956).

The following drugs were also investigated for their ability to inhibit the destruction of substance P: BrLSD, ergometrine, mescaline, eserine, chlorpromazine and strychnine. As may be seen in Table I, the only one of these drugs which inhibited the destruction of substance P was LSD.

Guinea-pig brain extract appeared to be incapable of destroying oxytocin. The oxytocin activity was measured on the rat uterus. The brain extract produced a small increase in the effect of oxytocin on this preparation. The oxytocin equivalent of the mixture before incubation was 120% of the theoretical value and this figure was not significantly changed by incubation for 1 or 2 hr. In a parallel experiment substance P was about 50% inactivated in 2 hr.

TABLE I

COMPARISON OF THE INFLUENCE OF VARIOUS DRUGS ON THE RATE OF DISAPPEARANCE OF SUBSTANCE P INCUBATED WITH ACETONE DRIED POWDERS OF GUINEA-PIG BRAIN

The % activity remaining after incubation is given in terms of a substance P standard. Nil indicates that substance P was incubated with brain extract in the absence of any drugs.

Drug	% Activity Remaining After Incubation
LSD 10^{-8}	100
BrLSD 10^{-7}	70
Nil	73
Strychnine 10^{-6}	73
Morphine 10^{-6}	75
Eserine 10^{-6}	74
Nil	75
Chlorpromazine 10^{-6}	62
Nil	60
Ergometrine 10^{-7}	74
Nil	73
Mescaline 10^{-6}	74
Nil	71

Incubation of LSD and substance P with chymotrypsin in place of guinea-pig brain extract showed that LSD offered no protection to substance P against destruction by chymotrypsin. After 30 min., the residual activity was estimated as 60% both when LSD was present and when it was absent. After 60 min., the estimates were 32% and 30% respectively.

BrLSD resembles LSD in its antagonism to 5-HT, but differs from it in that it does not have the same psychological effects (Cerletti and Rothlin, 1955), and may even antagonize those of LSD (Ginzel and Mayer-Gross, 1956). It was therefore tested for its effects on substance P. When it was directly applied to guinea-pig ileum in concentrations up to 10^{-6}, it neither increased nor decreased the response to substance P. When added to brain extract, it did not alter the effect of this extract on substance P, but when both LSD and BrLSD were present the inhibitory effect of LSD on the enzyme was prevented, so that substance P was inactivated. Evidence for this is shown in Fig. 4.

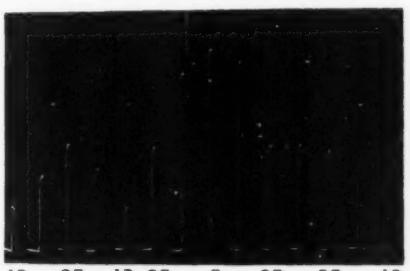

FIG. 4.—Guinea-pig ileum. 3 ml. bath. Responses to substance P after incubation with brain extracts (A, B, C_1, and C_2) compared with those to a standard preparation of substance P, the dose of which in units is indicated by the numerals. At A, 0.5 units (0.2 ml.) without an inhibitor. Note partial destruction. At B, 0.5 units with LSD 2×10^{-9}. Inhibition of destruction is evident. At C_1 0.55 units and at C_2 0.5 units each with LSD 2×10^{-9} and BrLSD 5×10^{-9}. There is now inhibition of the inhibition.

In this experiment, substance P was added to the brain extract in a concentration of 2.5 units/ml. Duplicate estimates of the substance P equivalent after incubation without inhibitors were 0.86 and 0.93 units/ml. In the presence of LSD (2×10^{-9}) the estimates were 2.1 and 2.3. In the presence of both LSD (2×10^{-9}) and BrLSD (5×10^{-9}) the estimates were 1.1 and 1.1. The inactivation was thus almost completely inhibited by LSD and this inhibition was mostly inhibited by BrLSD.

DISCUSSION

It seems evident from these results that LSD can protect substance P from enzymatic destruction. This is not due to the action of LSD on cholinesterase (Thompson, Tickner and Webster, 1955) since eserine does not have a similar action on the destruction of substance P. The specificity of LSD as an inhibitor of the destruction of substance P is noteworthy; BrLSD and ergometrine are inactive. A similar specificity is encountered in their activity as hallucinogens.

The evidence that BrLSD is an antagonist to LSD, both *in vivo* and *in vitro*, suggests that the observed relationship between substance P and LSD may be related to the effects of LSD on the brain.

Various attempts have been made to detect an effect of substance P on the brain. According to Pernow (1955) the cerebral intraventricular injection of substance P can cause rage in otherwise mild-tempered cats. In a single experiment of the same sort, Gaddum and Krivoy (unpublished observation) were unable definitely to confirm this observation, even by employing doses up to 500 units. However, more evidence must be obtained on this point before a definite conclusion can be reached. In the extensive experiments of Zetler (1956), the effect of substance P on the motor activity of mice was generally sedative.

This discussion of the relationship of these *in vitro* findings to *in vivo* observations is complicated by the fact that the effect of LSD on the destruction of substance P is lessened by increasing the duration of incubation. This may perhaps be due to destruction of LSD, but there is no direct evidence that LSD was destroyed in these experiments. The effect of LSD was also diminished

when large amounts of brain extract were used. This may have been due either to the destruction of LSD or to the fact that the total amount of LSD then became inadequate to block a sufficient number of receptors in the enzyme.

This work was undertaken during the tenure of a Public Health Service Postdoctoral Research Fellowship of the National Institute of Neurological Diseases and Blindness, Bethesda, Maryland, U.S.A.

The author wishes to express his gratitude to Professor J. H. Gaddum and the members of his department for their interest and invaluable aid in the completion of this investigation, as well as for their generous hospitality which made this research possible.

References

Amin, A. H., Crawford, T. B. B., and Gaddum, J. H. (1954). *J. Physiol.*, **126**, 596.

Cerletti, A., and Rothlin, E. (1955). *Nature, Lond.*, **176**, 785.

Ginzel, K. H., and Mayer-Gross, W. (1956). Ibid., **178**, 210.

Gullbring, B. (1943). *Acta physiol. scand.*, **6**, 246.

Pernow, B. (1953). Ibid., **29**, supp. 105.

—— (1955). *Z. Vitam. Horm. u. Fermentforsch.*, **7**, 59.

Rothlin, E. (1956). *Nature, Lond.*, **178**, 1400.

Thompson, R. H. S., Tickner, A., and Webster, G. R. (1955). *Brit. J. Pharmacol.*, **10**, 61.

Zetler, G. (1956). *Arch. exp. Path. Pharmak.*, **228**, 513.

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HISTAMINE RELEASE AND PAIN PRODUCTION BY XANTHOSINE AND RELATED COMPOUNDS

BY

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(RECEIVED MAY 22, 1957)

Xanthosine (5 to 50 μg.) caused pain on intradermal injection in all human subjects tested: in about half the subjects it also gave a flare. Inosinic acid, uric acid, allantoin, alloxan and theophylline caused pain, but not a flare by intradermal injection in man. Xanthine caused both pain and flare. A number of related compounds in similar doses caused neither pain nor flare. Inosine, inosinic acid, and guanosine, but not xanthosine, were powerful histamine liberators in animals. The lowering of the cat blood pressure, depression of the activity of the guinea-pig ileum and stimulation of guinea-pig uterus by adenosine were confirmed.

The purine nucleosides have recently been found effective in "reviving" stored blood (Prankerd and Altman, 1954; Gabrio, Donohue, and Finch, 1955; Prankerd, 1956a). Our interest in these substances arose from the need to decide which was the least pharmacologically active and hence most suitable for "reviving" blood intended for transfusion into patients. We concluded that guanosine was the most satisfactory, and it has in fact been used (Prankerd, 1956b).

In addition to repeating some of the earlier work, as reviewed by Drury (1936), on the cat blood pressure preparation, the isolated guinea-pig ileum and the isolated guinea-pig uterus, we tested the histamine-liberating properties of these compounds. Some of the nucleosides, especially inosine, were found to be potent histamine liberators. This suggested an investigation into their ability to increase capillary permeabiltiy and in most cases the results obtained paralleled those from estimation of histamine release: a full report of the capillary permeability experiments is given elsewhere (Spector and Willoughby, 1957).

An intradermal injection technique was then used to investigate the histamine liberating properties of these substances in man. We were surprised to find that xanthosine, which is only a very weak histamine liberator in animals, was a potent liberator, as judged by the production of a flare and weal, in about half the fifty subjects tested. Inosine, on the other hand, which liberated much histamine in animals, was inactive when injected intradermally in man.

Another unexpected finding was that xanthosine, on intradermal injection, caused a sharp pain in all subjects tested, and that this was not antagonized by an antihistamine (mepyramine maleate).

We conclude that xanthosine and some related compounds must be added to the list of naturally occurring pain-producing substances. The pain-producing properties are not antagonized by mepyramine maleate, although many of these compounds are histamine liberators in certain species. Some of our results were demonstrated to the British Pharmacological Society in January, 1957.

METHODS

The following preparations used for the present work were set up in the usual manner: (*a*) Cat blood pressure preparation. Anaesthetic: pentobarbitone. (*b*) Isolated guinea-pig ileum preparation. 18 ml. bath of Tyrode solution at 35°. (*c*) Isolated virgin guinea-pig uterus preparation. 18 ml. bath of Tyrode solution at 35°. (*d*) Rat hind-quarter preparation, perfused with Locke solution (Rocha e Silva and Schild, 1949). Full technical details of this preparation are given elsewhere (Willoughby, 1957). (*e*) Animals pretreated with trypan blue as described by Spector (1951). A 1% solution of trypan blue was injected intravenously into rats (1 ml.), rabbits (5 ml.), guinea-pigs (2 ml.), and mice (0.25 ml.).

In man, an intradermal injection technique was used in which the assessment of pain production was necessarily subjective, but conditions were kept as constant as possible. Injections were made intradermally with a 1-ml. tuberculin syringe through a 26-gauge

needle. All injections were made into the flexor aspect of the forearm. The volume of each injection was kept constant (0.05 ml.) and all solutions were at neutral *p*H and were made isotonic with 0.85% (w/v) NaCl. The procedure suggested by Armstrong, Dry, Keele, and Markham (1953) was used in which the needle was inserted for 15 sec. before the solution was injected. This eliminated the initial sensation of the insertion from the pain assessment. An initial injection of normal saline was made and the subject told that this was only saline and was to be compared with subsequent injections. All injections, except the first one of saline, were made in random order and the subject was not told what was being injected. Three or four injections after the first were made: these consisted of normal saline, histamine (0.05 μg.), xanthosine and sometimes other substances under test. After each injection the subject was asked if it had caused pain, and if so whether it was more or less severe than that produced by normal saline. The injection sites were observed after 10 min. and the presence or absence of a flare was noted. The criterion "a flare after 10 min." was adopted because: (*a*) An immediate flare, possibly associated with trauma, followed the injections in some subjects, but this rapidly faded over 5 min. Reading at 10 min. excluded this effect from the results. (*b*) Although a flare at 10 min. was usually associated with a weal obviously larger than that seen when saline had been injected, any intradermal injection led to formation of a small bleb which is occasionally difficult to distinguish from a weal. This difficulty was avoided by adopting the presence or absence of a flare as the criterion. (*c*) The response to the injection of histamine, and in some subjects to xanthosine and xanthine, was maximal after about 10 min.

A few subjects (five out of the first hundred) gave a very small flare at 10 min. to the control saline injection, but the response to xanthosine and xanthine was either the same as that to saline or obviously much larger.

A small number of additional experiments were performed in which injections were made into areas of skin pre-treated in various ways. The effect of local antihistamine was tested on six occasions by preparing the skin with mepyramine maleate (Anthisan) cream at intervals for 48 hr. before injecting xanthosine. On one occasion an area of skin depleted of histamine was produced by daily injections with 48/80 (1 μg.) until this no longer produced a triple response. On four subjects the antagonistic effects of procaine (1 ml. of 0.5% solution) were tested by subcutaneous infiltration a few minutes before the xanthosine was injected. In all experiments involving pre-treatment with drugs the untreated arm was used as control.

Chemicals Used.—The values for histamine are expressed as base, but the doses given for the other substances refer to the following: adenosine, cytosine, guanosine, inosine, adenine, cytidine nitrate, guanidine hydrochloride, hypoxanthine, guanylic acid sodium salt, theophylline, caffeine, alloxan, allantoin, uric acid (all the foregoing were obtained from Light); theobromine (British Drug Houses); mepyramine maleate (Anthisan, May and Baker). Compound 48/80 as chloride was kindly given to us by Burroughs Wellcome, and inosinic acid by Sigma Chemical Co., U.S.A. Paper chromatography of the nucleosides did not reveal any impurity other than a 10% contamination by the corresponding purine base.

Results

Effects on Cat's Blood Pressure Preparation.—Adenosine (1 mg./kg.) gave either a depressor or biphasic type of response which was not abolished by hexamethonium (25 mg./kg.) or atropine (0.1 mg./kg.). This depressor response was not antagonized by mepyramine maleate (5 mg./kg.) and this is evidence against the possibility that histamine liberation is responsible for the fall in blood pressure. An infusion of 5 mg. of adenosine over 5 min. produced a drop in blood pressure of 25 mm. of mercury.

In similar doses inosine had no effect, but the response to doses of 10 mg./kg. was very similar to that of 1 mg./kg. adenosine. This agreed with the results of Green and Stoner (1950).

Guanosine, infused in doses of 2.5 mg./kg. and 25 mg./kg., did not alter the blood pressure. Single doses of 20 mg./kg. had no effect. Xanthosine, cytidine, guanylic acid, hypoxanthine and cytosine were all without effect on the cat blood pressure in doses up to 5 mg./kg.

In one experiment an electrocardiogram was taken and the effect of the purine nucleosides was tested in doses of 2.5 mg./kg. Adenosine, the only nucleoside to affect the blood pressure in this dosage, caused a nodal rhythm. The remainder had no effect.

Effects on Guinea-pig Ileum.—The purine bases, nucleosides and nucleotides were tested on the isolated guinea-pig ileum. Adenosine had a relaxant action on the isolated gut and antagonized histamine-produced contractions. In doses of 0.1 mg. adenosine reduced the response to 0.05 μg. histamine by at least 50%. Contractions produced by acetylcholine were also antagonized by doses of 0.1 mg. adenosine and recovery was slow after washing. Guinea-pig ileum does not normally show spontaneous activity, though it may occasionally do so. Adenosine was added to a spontaneously active preparation on one occasion and caused relaxation with inhibition of the contractions.

Guanosine, guanidine, guanylic acid, adenine, inosine, xanthine, xanthosine, hypoxanthine, cytidine or cytosine all had no effect on the isolated ileum in doses up to 0.25 mg.

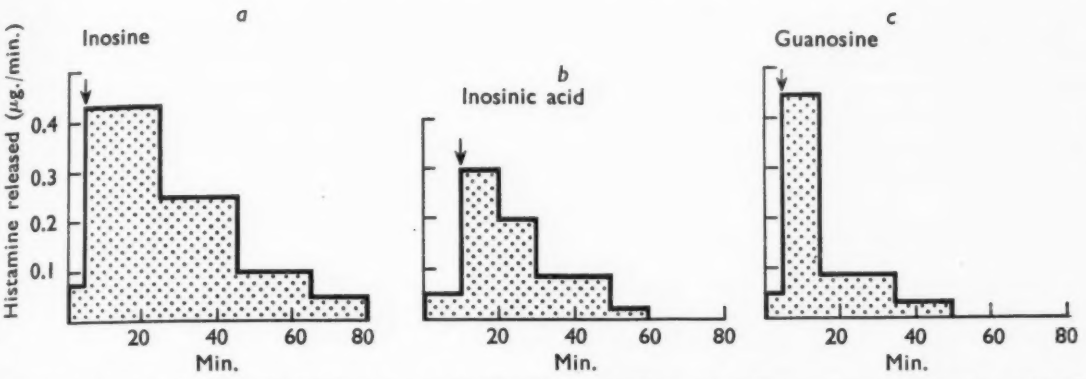

FIG. 1.—Histamine release (ordinates) from the rat hind-quarters preparation. (*a*), 0.25 mg. of inosine liberated a total of 14.9 μg. of histamine. (*b*), 0.25 mg. of inosinic acid liberated a total of 5.0 μg. of histamine. (*c*), 0.25 mg. of guanosine liberated a total of 5.0 μg. of histamine.

Effect on Guinea-pig Uterus.—The nucleosides were tested on the isolated guinea-pig uterus preparation. 0.25 mg. of adenosine produced a contraction approximately equal to that following 0.01 μg. of acetylcholine. Xanthosine, inosine and guanosine had no effect in doses of up to 1 mg.; they did not affect contractions elicited by acetylcholine. These results are in agreement with the findings of von Euler and Gaddum (1931) and Flossner (1934).

Rat Hind-quarters Perfusion.—The histamine liberated was assayed on the guinea-pig ileum preparation and its identity as histamine was established by parallel inhibition experiments using mepyramine maleate on both the isolated guinea-pig ileum and cat blood pressure preparations. Inosine was found to be a fairly potent histamine liberator: 0.25 mg. always released 12 to 15 μg. of histamine. The release was of an explosive type (Fig. 1) similar to that reported with other liberators (MacIntosh and Paton, 1949; Feldberg and Paton, 1951). The release of histamine by inosine appears to be greater than that by (+)-tubocurarine as reported by Rocha e Silva and Schild (1949) using a similar preparation. Guanosine was less active, 0.25 mg. of guanosine releasing only 5.0 μg. of histamine. Xanthosine, adenosine and cytosine in the same dosage released negligible quantities of histamine (<1 μg.) from this preparation. A larger dose of xanthosine (10 mg.) liberated 5.2 μg. of histamine. Inosinic acid (0.25 mg.), which is the nucleotide corresponding to inosine, liberated 4.5 μg. histamine. Guanylic acid gave no liberation, nor did doses of 0.25 mg. of the bases guanidine, xanthine, adenine, hypoxanthine or cytidine. Hypoxanthine (0.25 mg.), which is the base corresponding to inosine, gave a small liberation (0 to 2.5 μg. histamine). All the substances tested were injected at least twice on separate preparations at approximately neutral *p*H. After any compound which released little or no histamine, an injection of 10 μg. of 48/80 always resulted in a large release, proving the ability of the preparation to liberate histamine. Preparations which became grossly oedematous were discarded, since the oedema fluid pressed out of such a preparation after a large dose of 48/80 was found to contain the same quantity of histamine as the perfusate.

Trypan Blue Method for Capillary Permeability.—Table I shows the effect of intradermal injection of the nucleosides on capillary permeability in animals. Inosine was very active on all the species tested; guanosine and adenosine had some effect, but xanthosine was without effect. This method has been used in the rat to test a large number of substances related to inflammation, including the nucleic acid derivatives, for the property of increasing capillary permeability (Spector and Willoughby, 1957).

TABLE I

THE EFFECT OF PURINE NUCLEOSIDES INCREASING CAPILLARY PERMEABILITY IN THE SKIN OF LABORATORY ANIMALS

In this and subsequent tables, the presence of a response is denoted by +. The more intense the response, the more symbols + are recorded. 0 denotes the absence of a response. ± indicates a threshold response.

Substance	Increase in Capillary Permeability following Intradermal Injection of 25 μg. of Nucleoside			
	Rat	Mouse	Guinea-pig	Rabbit
Xanthosine	None	None	None	None
Inosine ..	+++	+++	+++	+++
Guanosine	+	±	+	±
Adenosine	+	±	+	+

Intradermal Injections in Man

Flare Production.—Twenty-four of an initial group of fifty subjects tested with 50 μg. of xanthosine gave a marked flare round the injection site equal to or larger than that from 0.05 μg. of histamine. Subjects giving a flare to xanthosine will be referred to as " reactors " and those not giving one as " non-reactors."

The response of a reactor to xanthosine (50 μg.) was indistinguishable from that to histamine (0.05 μg.), but the weal component of the response has been disregarded for reasons given in the method. Four doses of xanthosine was tested in a group of 15 subjects (Table II). Seven reactors gave a flare

TABLE II

RESPONSE IN MAN TO INTRADERMAL INJECTIONS OF XANTHOSINE

For assessment of responses see Table I.

Dose (μg./ 0·05 ml.)	Reactors to Xanthosine		Non-reactors to Xanthosine	
	Flare	Pain	Flare	Pain
5	+	+	0	+
20	+	++	0	++
30	+	+++	0	+++
50	+	++++	0	++++
No. of subjects	7		8	

but eight non-reactors gave no flare with any dose tested. The size of flare in the reactors increased with increasing dose. The division into reactors and non-reactors was sharp: a reactor always gave a typical flare even with 5 μg., whilst a non-reactor did not, even with 50 μg. Xanthine has only been tested in a dose of 50 μg.

The effect of pre-treatment of the skin with an antihistamine or a local anaesthetic is shown in Table III. In six reactors, mepyramine maleate cream was found to prevent the appearance of the flare to 50 μg. xanthosine. One reactor had an area of skin depleted of histamine by injections of 48/80; subsequent injections of xanthosine into the area then failed to produce a flare. The pre-treatment with 48/80 was very painful and has not been repeated. Procaine infiltration prevented the flare component of the response, but the weal was not affected.

The nucleosides adenosine, inosine and guanosine did not give a flare even when the dose was increased to 250 μg. (Neither did 0.05 ml. of a saturated solution of guanosine, 20°.) Xanthinic acid was not available; guanylic and inosinic acids gave no triple response. The base xanthine gave results identical to those obtained with xanthosine, but adenine, guanine and hypoxanthine were inactive. None of the related compounds shown in Table IV gave a flare on intradermal injection. Dosage of many compounds was limited by solubility.

Pain Production.—All of the fifty subjects given intradermal injections of 50 μg. of xanthosine complained of pain. This was a sharp, " bright " pain of immediate onset, which lasted only a few seconds and which began to decline in severity as soon as the injection was completed. With thirty of these subjects the procedure was repeated using 5 μg. of xanthosine. All the subjects reported that injection of normal saline was painless when compared with 50 μg. of xanthosine and that 5 μg. of xanthosine was definitely more painful than the saline. The fifteen subjects given doses ranging from 5 to 50 μg. (Table II) were asked to place the injections in order of increasing pain. Three of them placed the 20 and 30 μg. injections in reversed order, but otherwise the placing according to severity of pain corresponded with the dose.

Pain production was quite independent of the presence or absence of a flare. Non-reactors seemed to suffer just as much pain as reactors. The results are shown in Table IV.

The effects of various pre-treatments are shown in Table III. Local anaesthesia with procaine made subsequent injections of 50 μg. of xanthosine completely painless, although in reactors a weal was still produced. Inunction with sufficient antihistamine to prevent the formation of a flare in reactors had no effect whatever on the pain

TABLE III

MODIFICATION OF THE RESPONSE TO INTRADERMAL INJECTION OF 50 μG. OF XANTHOSINE IN MAN BY PROCAINE, BY 48/80 AND BY MEPYRAMINE

For assessment of responses see Table I.

Pre-treatment	No. of Expts.		Flare		Pain	
	Reactors	Non-reactors	Reactor	Non-reactor	Reactor	Non-reactor
None	24	26	++++	0	++++	++++
Procaine infiltration ..	2	2	Weal only	0	0	0
Histamine depletion ..	1	0	0		++++	
Mepyramine maleate ..	6	0	0		++++	

TABLE IV

RESPONSE IN MAN TO INTRADERMAL INJECTIONS OF THE NUCLEOSIDES AND SOME RELATED COMPOUNDS

For assessment of responses see Table I.

Substance	Dose (μg./0·05 ml.)	Reactors to Xanthosine		Non-reactors to Xanthosine		No. of Subjects		
		Flare	Pain	Flare	Pain	Total	Reactors	Non-reactors
Xanthosine	50	+	++++	0	++++	50	24	26
Adenosine	50	0	0	0	0	15	8	7
"	250	0	0	0	0	6	3	3
Inosine	50	0	0	0	0	15	7	8
"	250	0	0	0	0	6	3	3
Guanosine	50	0	0	0	0	15	8	7
"	Saturated solution	0	0	0	0	6	3	3
Xanthine	50	+	++++	0	++++	6	3	3
Adenine	50	0	0	0	0	6	3	3
Hypoxanthine	50	0	0	0	0	6	3	3
Guanine	50	0	0	0	0	6	3	3
Guanylic acid	50	0	0	0	0	6	3	3
Inosinic "	50	0	+++	0	+++	6	3	3
Uric acid	Saturated solution	0	+++	0	+++	12	6	6
Allantoin	50	0	+	0	+	6	3	3
Alloxan	50	0	+++	0	+++	6	3	3
Uracil	250	0	+	0	+	6	3	3
Caffeine	50	0	0	0	0	6	3	3
Theobromine	50	0	0	0	0	12	6	6
Theophylline	50	0	+	0	+	6	3	3
"	250	0	+++	0	+++	9	6	3
Histamine	0·05	+	Itch	+	Itch	50	24	26

from xanthosine. Similarly in the single histamine depletion experiment subsequent xanthosine injections were just as painful as before.

The other nucleosides did not cause pain in any dose tested (up to 250 μg.). Of the bases, only xanthine caused pain on intradermal injection, and this pain was just as severe, and of a similar quality to that from xanthosine. Adenine, guanine, and hypoxanthine did not cause pain. Alloxan (50 μg.), theophylline (250 μg.), inosinic acid (250 μg.) and a saturated solution of uric acid (approximately 2.5 μg.) all caused less pain than 50 μg. of xanthosine. Allantoin (50 μg.) and uracil (250 μg.) only caused slight pain. Guanylic acid (50 μg.), caffeine (50 μg.), and theobromine (50 μg.) were not painful on intradermal injection.

With none of the substances tested was there any correlation between the production of a flare and the sensation of pain. In the concentration used, histamine caused itching rather than pain. No itching was felt after injections of any of the nucleosides or related compounds.

DISCUSSION

The depression of the cat blood pressure by inosine and guanosine was unlikely to be due to histamine release as it was not affected by mepyramine. Although inosine and, to a lesser extent, guanosine were powerful histamine liberators in animals, but not in man, xanthosine was only a very weak liberator in animals but gave a flare in about half of the human subjects. This suggests that if drugs for use in man are only tested on laboratory animals, it is possible for a histamine liberator to be overlooked. In about half the human subjects, xanthosine (50 μg.) gave a response indistinguishable from that to histamine (0.05 μg.). This response was antagonized by mepyramine and in a single experiment did not appear after histamine depletion. Hence it is concluded that the flare and weal produced by xanthosine in some subjects probably result from histamine liberation. In 50 experiments we have not found a reactor change to a non-reactor or vice versa when injections of xanthosine were repeated. Reactors and non-reactors have been classified on the basis of their reaction to xanthosine, but an identical response was obtained with xanthine in a smaller group of subjects.

Xanthosine, xanthine and uric acid are all naturally occurring, powerful pain-producing substances. The pain seems very unlikely to be dependent upon histamine release, since they hurt reactors and non-reactors equally and the pain produced by histamine is of a quite different quality. In a preliminary experiment, local pre-treatment with subcutaneous injection of diphenhydramine hydrochloride (10 mg.) was performed, but this was very painful and was replaced by inunction with mepyramine maleate. Pre-treatment with these antihistamines never gave any decrease in the pain following xanthosine injections, and this is further evidence against the pain being dependent upon histamine release. The effect of pre-treating the skin with procaine

and mepyramine on the pain produced by substances other than xanthosine has not been investigated.

Some of these compounds might be involved in pain production following tissue damage, and their abnormal metabolism and accumulation could account for some of the pain in gout.

We cannot offer an adequate explanation of the division into reactors and non-reactors. A natural or acquired hypersensitivity to xanthosine or xanthine on the part of the reactors seems the likeliest explanation which is supported by the highly antigenic properties of many compounds derived from a pyrimidine nucleus (Alexander, 1955). The genetical aspects of the division are at present being investigated.

We are unwilling to attempt to relate pain production in this group of substances to their chemical formulae as thorough investigation of the group by this technique requires injection of many subjects with a wide range of doses. However, it is of interest that six out of the eight compounds causing pain on intradermal injection have oxygen atoms linked to carbon 2. On the other hand, of the nine substances tested which did not cause pain, only two have this grouping.

The estimation of pain is necessarily subjective, but the pain due to xanthosine is of a different quality to that from acetylcholine, histamine or 5-hydroxytryptamine, none of which gave such an intense "bright" pain. The differences in quality make comparison of dose responses very difficult and this has not been attempted.

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References

Alexander, H. L. (1955). *Reaction with Drug Therapy*, p. 13. Philadelphia: Saunders.

Armstrong, D., Dry, R. M. L., Keele, C. A., and Markham, J. W. (1953). *J. Physiol.*, **120**, 326.

Drury, A. N. (1936). *Physiol. Rev.*, **16**, 292.

Feldberg, W., and Paton, W. D. M. (1951). *J. Physiol.*, **114**, 490.

Flossner, O. (1934). *Arch. exp. Path. Pharmak.*, **174**, 245.

Gabrio, B. W., Donohue, D. M., and Finch, C. A. (1955). *J. clin. Invest.*, **34**, 1509.

Green, H. N., and Stoner, H. B. (1950). *Biological Actions of the Adenine Nucleotides*, p. 69. London: Lewis.

MacIntosh, F. C., and Paton, W. D. M. (1949). *J. Physiol.*, **109**, 190.

Prankerd, T. A. J., and Altman, K. I. (1954). *Biochem. J.*, **58**, 622.

—— (1956a). Ibid., **64**, 209.

—— (1956b). *Lancet*, **1**, 469.

Rocha e Silva, M., and Schild, H. O. (1949). *J. Physiol.*, **109**, 448.

Spector, W. G. (1951). *J. Path. Bact.*, **63**, 93.

—— and Willoughby, D. A. (1957). Ibid., **73**, 133.

Von Euler, U. S., and Gaddum, J. H. (1931). *J. Physiol.*, **72**, 74.

Willoughby, D. A. (1957). *J. Inst. Sci. Techn.*, **3**, 23.

Brit. J. Pharmacol. (1957), **12**, 371.

DIGOXIN, OUABAIN AND POTASSIUM MOVEMENTS IN RABBIT AURICLES

BY

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Movements of potassium have been observed in isolated right and left auricles of rabbits by means of radioactive tracer. The tissues have been immersed in a modified Krebs saline at 37°, and in these conditions showed only small changes in ionic content over periods up to 6 hr. The extracellular volume, determined with inulin and with ^{24}Na, was large (44.1 ml./100 g. tissue), and accounted for about 3.8% of the tissue potassium. Left auricles exchanged without gross inhomogeneity: the rate of exchange was about 1.5% of the total tissue potassium/min., though it was probably higher in the first half-hour or hour after preparation. In right auricles the exchange was less homogeneous and included a faster component than that observed in left auricles. Digoxin and ouabain reduced the influx and had no appreciable effect on the efflux of potassium, so that the auricles lost potassium. The threshold concentrations which produced effects within 20 min. were of the order of 10^{-6} for ouabain and somewhat higher for digoxin. Irregularities or failure of contraction occurred when the tissue potassium was reduced by about 15%. Loss of potassium was accompanied by gain of sodium, but the tissue appeared unsuitable for making estimations of the rate of sodium movement.

Hearts are generally less sensitive to cardiac glycosides when they are in a medium rich in potassium (Clark, 1912; Baker, 1947; Friedman and Bine, 1947; Lown, Salzberg, Enselberg and Weston, 1951; Hazard, Hazard, and Thouvenot, 1956), and these drugs, at least in toxic doses, cause loss of potassium from the heart muscle (Calhoun and Harrison, 1931; Wood and Moe, 1938; Wedd, 1939; Friedman and Bine, 1947; Hajdu, 1953; Regan, Talmers, and Hellems, 1956), and diminish the rate of exchange of potassium (Conn, 1956). It is therefore likely that the potassium influx is diminished, as in erythrocytes (Schatzmann, 1953) and other tissues, but it is less obvious whether the efflux is modified. These fluxes have therefore been estimated in rabbit auricles, and the effects of digoxin and ouabain on them have been observed.

METHODS

Saline.—Unless otherwise stated, the salt solution used contained Na^+ 146 mM, K^+ 5 mM, Ca^{++} 1.7 mM, Mg^{++} 1.2 mM, Cl^- 124 mM, HCO_3^- 25 mM, SO_4^{--} 1.2 mM, $H_2PO_4^-$ 1.2 mM, dextrose 0.2% (w/v) and was equilibrated during use with 95% O_2+5% CO_2. When appropriate, part of the Na or K was replaced by ^{24}Na or ^{42}K. The radioactive salts were prepared from spectroscopically pure Na_2CO_3 and K_2CO_3 which had been exposed to neutron bombardment at A.E.R.E., Harwell, and was converted to the chlorides by addition of N HCl and sufficient water to make neutral 154 mM solutions.

Auricles.—Young rabbits, mostly weighing 1.0 to 1.5 kg., were concussed and bled from the carotid arteries. The heart was removed from the chest and rinsed in saline, and the auricles were freed by dissection partly in air and partly in saline at room temperature. In some experiments they were used free and in others they were attached by nylon threads to a frame so that most of the auricle was stretched as a flat sheet: surplus tissue was then cut away. In most experiments left and right auricles were studied separately; the right generally beat spontaneously, while the left did not beat, or did so at infrequent intervals. A few experiments were performed with pairs of auricles suspended so as to beat isotonically, when the contractions were recorded on smoked paper.

Measurement of ^{42}K Influx and Changes in Total K and Na Content.—Free or stretched auricles were immersed in tubes containing 15 ml. radioactive saline medium in a water bath at 37°. After various periods, the auricles were removed, rinsed with 0.15 M choline chloride to remove saline adhering to the outside of the tissue, weighed, dried for 2 hr. or overnight at 105° and weighed again. The dry tissue was ashed with 0.5 ml. conc. HNO_3 for 1 to 2 hr., followed by a further hour after dilution with 5 ml. H_2O. The solution was made up to 25 or 50 ml. for estimation

of potassium and sodium by means of a flame photometer and ^{42}K by means of a liquid counting tube and conventional scaler and power supplies. Calculation of the rate of influx from these results is described in connexion with the results. Alternatively, an estimate of the rate of influx was obtained at the same time as one of the efflux as described below.

Measurement of ^{42}K Efflux.—Stretched auricles were immersed in radioactive saline medium for various periods and then transferred to an apparatus similar to that described by Creese (1954) in which the tissue was perfused with oxygenated inactive saline at 37° while it lay over a shielded Geiger tube separated by a window of Perspex (polymethylmethacrylate resin) approximately 0.15 mm. thick. The decline in radioactivity was followed by counting from time to time as required. At the end of the experiment the auricle was treated as described above and its specific activity was compared with that of the saline in which soaking-in of tracer had occurred. The sensitivity of the efflux counter was determined from the final count of the efflux bath, the ratio of specific activities of auricle and saline when counted in a liquid counter, and the potassium content of the auricle, so that the total potassium entry during the soak-in period could be determined.

As there was some uncertainty about the adequacy of counts made in this way on beating tissue, efflux was sometimes followed by placing the auricles in a bath containing 50 ml. of inactive medium and replacing the medium after 1, 2, 5, 10 min. and then every 5 or 10 min. for 1 to 4 hr. The radioactivity of each sample of medium was then determined with a liquid counter.

Estimations of Radioactivity.—All estimates were corrected for background, resolving time of the counting equipment used, and decay, the half-time for ^{42}K being taken as 12.4 hr. and for ^{24}Na as 15.1 hr. The activity of standard solutions was checked and found to be consistent with these values for at least six half-lives; all experiments were completed within this time.

Inulin Space.—The inulin spaces were estimated by immersing auricles in saline containing 1.0% inulin (w/v) for 60 min., blotting lightly, rinsing with an isotonic solution and blotting again before transfer to an inulin-free saline for a second hour. The inulin was estimated in both solutions by the method of Bacon and Bell (1948), and the sodium, potassium, and water content of the auricles was determined as usual. Estimations were made on free and stretched left and right auricles and on isotonically contracting pairs of auricles.

Cell Dimensions.—Auricles were cooled or were poisoned with ouabain until they ceased to beat and were then fixed in saline containing 10% (v/v) of neutralized formalin, and embedded in polyethyleneglycol-1000 monostearate (Miles and Linder, 1952). Sections were stained with haematoxylin and van Gieson, magnified ×1,000 and projected on a screen so that the diameter of the cells could be measured.

Drugs.—Pure crystalline ouabain and digoxin were used as supplied (Burroughs Wellcome) in concentrations (w/v) stated in the description of results. Digoxin was dissolved in 70% ethanol before addition to the saline, so that the final solutions contained up to 0.5% ethanol. Control observations were made when appropriate with saline containing the same concentrations of ethanol.

Results

Cell Dimensions.—Three divided pairs of auricles were used. One pair was cooled to stop beating and fixed as soon as it had been dissected. A second pair was treated similarly after 3 hr. immersion in the usual oxygenated saline; and the third pair was immersed for 50 min. in the same medium, to which ouabain (10^{-6}) had been added.

Sections of the six auricles were examined independently by two persons, each of whom measured the maximum width or diameter of 30 cells in each slide. The sections showed cells cut at various angles; in view of the arrangement of fibres in the myocardium, this was probably unavoidable. Where cells were cut predominantly longitudinally, so that opposite sides of the cell were approximately parallel, the distance between the walls at the widest point was taken as the cell diameter; with cells cut predominantly transversely, so that the cells appear oval or round on cross section, the minimum diameter was used. A number of areas were used on each slide, and the measurements were made on the best defined cells in each field. No means was devised for obtaining unbiased samples, and no correction was applied for possible shrinking during preparation. The mean diameter of 360 cells was 7.0 μ: the mean for left auricles was 6.2 μ and for right auricles 7.7 μ, each with a standard deviation of ± 2.7 μ. Analysis of variance showed the difference between left and right sides to be significant, but did not indicate appreciable significance in the effects of immersion or ouabain on the cell size. It was assumed that the cells were sufficiently nearly circular on cross section for the diameter so estimated to provide an adequate estimate both of circumference and of cross sectional area, and the ratio of these measurements, using 7.0 μ as diameter, was taken as the ratio of surface area to volume.

Composition of Auricles.—Observations on the sodium, potassium and water content of auricles immersed without stretching for various periods of time are shown in Table I. Right auricles were generally smaller and consistently contained less potassium, even with allowance for the difference in weight. There were no significant changes in

TABLE I
MEAN COMPOSITION OF UNSTRETCHED RABBIT AURICLES IN EXPERIMENTAL CONDITIONS

Condition	No. of Auricles L	No. of Auricles R	Wet Wt. (mg.) L	Wet Wt. (mg.) R	Dry Wt. (mg.) L	Dry Wt. (mg.) R	H_2O (g./kg. Dry Wt.) L	H_2O (g./kg. Dry Wt.) R	K^+ (mEq./kg. Dry Wt.) L	K^+ (mEq./kg. Dry Wt.) R
Dissected and rinsed	3	4	135·5	130·0	24·7	23·0	4·48	4·65	374	342
Immersed for 5 to 30 min.	7	6	127·8	96·1	20·2	16·9	5·33	4·69	350	325
,, ,, 120 ,,	6	6	135·3	95·7	24·4	18·2	4·61	4·28	332	308
,, ,, 360 ,,	3	3	142·9	123·7	26·6	23·4	4·37	4·29	299	290

the mean sodium and water content of the tissues with time, but there was a small, significant ($P<0.02$ left, <0.1 right) diminution in the potassium content on either side, at a rate of about 2% of the total K/hr. No loss of potassium occurred in a few experiments in which the concentration of potassium in the medium was 7.5 instead of 5 mM. The inulin spaces were surprisingly large, 2.40 ± 0.085 (S.E.) ml./g. dry weight of tissue, or 44.1 ml./100 g. wet weight. This high value was supported by observations, described later, on the efflux of ^{24}Na after a period of immersion in saline containing this tracer. Estimations of the intracellular ionic concentration by deducting the amounts expected to be present in the extracellular fluid are therefore rather unreliable. In the case of sodium, the mean deduction is about 85% of the total in the tissue, and the estimate so obtained is practically worthless: with potassium the deduction is much smaller (about 3.8%) and such variation as may occur in the extracellular volume is comparatively unimportant.

Stretching the auricles had no detectable influence on the composition of tissues which had been immersed for an hour or more. Observations made less than an hour after immersion gave unusually low values for potassium, but these observations were not comparable with those on unstretched tissues because the dissection and setting up of the preparation, which was done at room temperature, took longer (10 to 20 min. instead of 3 to 5 min. from the time of killing the animal to immersion) and the initial loss of potassium may have been due to cooling and manipulating rather than to an effect of stretch.

^{24}Na Efflux.—The large volume of the inulin space suggested that observations on the movement of ^{24}Na would give information mainly about the extracellular sodium. Some experiments were therefore done in which beating auricles were immersed for varying periods in saline medium containing ^{24}Na and were then transferred to an inactive medium, which was changed frequently and collected for counting after contact with the auricles. By adding the amounts of tracer lost in each portion of fluid to the amount remaining in the auricle at the end of the experiment, graphs were constructed to show the count of the tissue at different times (Fig. 1). These counts, plotted on semi-logarithmic paper, lay on a line which was considerably curved for at least 25 min. The shape of the curve was similar whether influx had proceeded for 5, 30 or 60 min. The estimate of the total tracer in the tissue at the beginning of the efflux was higher than would have occurred if the auricle Na had reached the specific activity of the medium; there was no evidence that the tissue Na had varied during the experiment, and the excess was probably due to small amounts of the highly active influx medium being carried over on the surface of the auricle and not removed because rinsing and blotting were less thorough at this rapid transfer than at the end of the experiment. It is therefore not directly shown how completely the tissue Na had exchanged at the beginning of the experiment, but the difference in position of the efflux curves after 5 or 60 min.

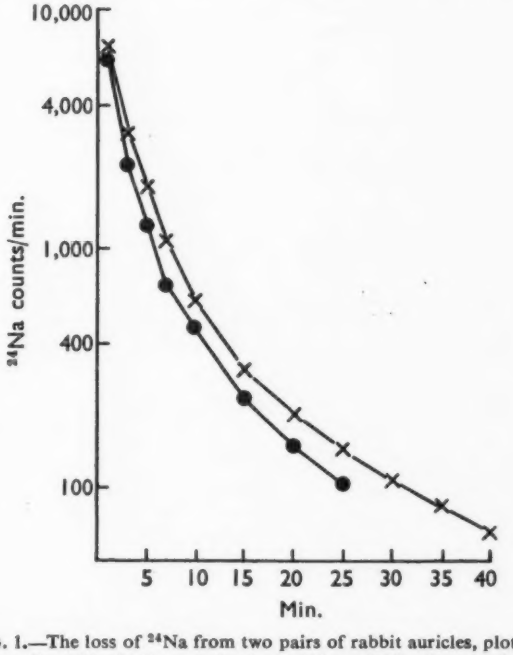

FIG. 1.—The loss of ^{24}Na from two pairs of rabbit auricles, plotted semilogarithmically. ●, 5 min. soak-in. X, 30 min. soak-in.

influx suggests that only a small additional amount of the total tissue Na exchanged after the first 5 min. The continued flattening of both curves makes it difficult to distinguish clearly between the exchange of intracellular and extracellular sodium, but retropolation of the later points suggests that the slowly exchanging, presumably intracellular fraction, accounts for less than 7 to 8% of the total tissue Na. This agrees with the results of inulin determinations and supports the large estimate of the extracellular space.

^{42}K Influx, Normal Auricles.—Measurements of influx were obtained by immersing auricles, usually unstretched in active saline, removing them at various times, and estimating their activity and potassium content after ashing. In some experiments, the auricles were immersed in active saline immediately after dissection. The results of six such experiments with 56 auricles are shown in Fig. 2. Left and right auricles behaved differently. In both, the uptake of ^{42}K did not follow the exponential course which would arise if the tissue potassium was uniformly exchangeable at a constant rate. The departure from an exponential course was greater in right auricles, which were generally beating spontaneously, than in left auricles: but in both the early part of the uptake was much more rapid than the late part. The rapid phase was too large to be due solely to the extracellular potassium, which accounts for less than 4% of the total in the tissue. Part of the apparent decline in rate is a consequence of averaging the uptake of a number of different auricles, which varied somewhat in size and probably in individual flux rates: the mean uptakes at different times from a population of single exponential uptake curves do not necessarily lie on a single exponential curve. However, there was also a real decline in the rate of uptake as the time of immersion increased, as was seen when auricles were immersed in inactive saline for 90 min. after dissection and then transferred to the active medium (Table II). Right auricles showed a much larger departure from a single exponential uptake, and a greater decrease in rate with time. This difference was not simply a consequence of beating, as the following experiment showed. Undivided pairs of auricles were immersed in active saline for various periods and were removed and then divided for drying, ashing and estimation. The pairs of auricles were observed to beat, but the uptake of radioactivity of the left auricles showed little difference from that of left auricles separated before immersion in the active medium (Table III). There was more difference between left and right auricles when they were separate than when they were undivided; division appeared both to accelerate the exchange in right auricles and to retard it in left auricles. None of the differences were large, and it is at least clear that the differences between left and right auricles did not

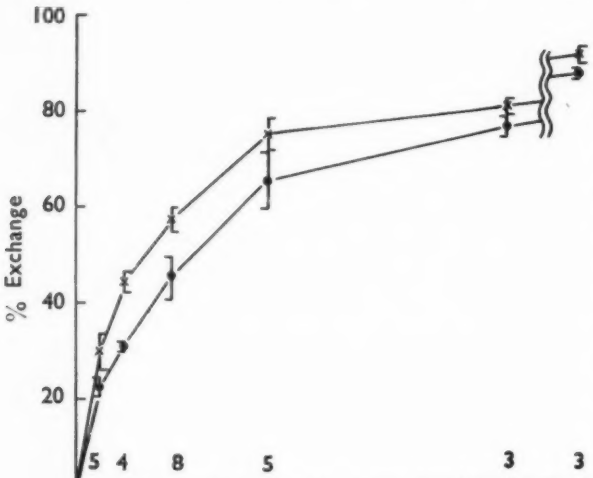

FIG. 2.—The uptake of ^{42}K by rabbit auricles. X, Right auricles. ●, Left auricles. The range marks show the standard errors of the mean. The figures above the abscissa show the number of observations on which each pair of points is based. The ordinate shows the specific activity of the auricles as % of that of the medium.

TABLE II

UPTAKE OF ^{42}K BY LEFT AND RIGHT AURICLES IMMERSED IN RADIOACTIVE SALINE IMMEDIATELY OR 90 MIN. AFTER DISSECTION

Left/right differences in exchange are highly significant ($t=4{\cdot}31$, $P<0{\cdot}01$).

Delay min.	Side	No. of Obs.	Dry Wt. (mg.) Mean±S.E.	K Content (mEq./kg. Dry Wt.) Mean±S.E.	K Exchanged After 20 min. (%) Mean±S.E.	Significance of Difference of Exchanges	
						t	*P*
0	Left	4	18·0±1·3	360±5	30·3±0·6	1·26	>0·3
90	,,	4	17·1±2·3	365±11	27·6±2·0		
0	Right	4	16·0±1·1	358±9	42·2±2·0	4·00	<0·01
90	,,	4	16·7±1·9	362±14	33·2±1·1		

TABLE III

UPTAKE OF ^{42}K BY LEFT AND RIGHT AURICLES IMMERSED IN RADIOACTIVE SALINE BEFORE OR AFTER DIVISION

Side	Condition During Immersion	No. of Obs.	Dry Weight (mg.) Mean±S.E.	K Content (mEq./kg. Dry Wt.) Mean±S.E.	% K Exchanged at End of Immersion Mean±S.E.	Significance of Difference	
						t	*P*
20 min. Immersion							
Left	Undivided	4	24·3±3·6	371±7	33·0±2·0	1·29	≃0·25
	Separate	4	18·0±1·3	360±5	30·3±0·6		
Right	Undivided	4	18·9±2·4	358±12	36·7±1·0	2·50	≃0·05
	Separate	4	16·0±1·1	358±9	42·2±2·0		
40 min. Immersion							
Left	Undivided	4	26·7±1·9	376±10	44·0±2·8	0·50	≃0·63
	Separate	4	21·9±2·1	342±7	42·0±2·9		
Right	Undivided	4	21·6±2·4	319±35	50·5±1·9	0·75	≃0·48
	Separate	3	15·8±0·7	281±22	54·2±4·6		

depend only on the inactivity of the separated left tissues. The longest period over which uptake was followed was 6 hr., by which time both left and right auricles had exchanged about 90% of their potassium (Fig. 2). In some experiments in a medium containing 7.5 mM K instead of 5.0 mM K, the exchange at this time was virtually complete (98.6%, mean of six observations: range 94.6 to 102.1%).

^{42}K Efflux, Normal Auricles.—The efflux of ^{42}K after a period of immersion in active saline was measured by repeated direct counting of the same auricle immersed in inactive saline, or by allowing efflux to occur in a bath of moderate volume, the contents of which was changed frequently and counted after removal from the tissue. With direct counting of the auricles, errors may arise from the change in geometry which occurs at each contraction. These errors were minimized by gently stretching the tissue on a frame and allowing contraction to occur isometrically; and such errors were minimal with the usually inactive, left, auricles. Anomalous results arise if pairs of auricles are used and cover an area substantially larger than the window of the counter, presumably because of the different rates of exchange of the two halves: and experiments of this sort have been disregarded. When efflux occurs in a bath whose contents is changed intermittently, some error results because the specific activity of the medium is not kept at zero; but with frequent changes, and a large bath as used here, this effect is trivial. Experiments have been done in both ways, and the reasonable agreement between the results suggests that neither source of error is important.

In some experiments with direct counting, the auricles have been transferred from the soak-in bath to the counting bath from time to time during the period of uptake, and their radioactivity and its initial rate of decline observed over three counts within 4 min. at each time. This procedure is inaccurate because it is difficult to ensure that the auricle is brought to exactly the same position in relation to the counting window each time, and small variations in position can have a large influence on the counting rate. However, the procedure gives a rough check on the rate of uptake of ^{42}K and the lability of what has been taken up. It is preferable to allowing the influx to occur in the same bath as the efflux, as it is difficult to be certain that the highly active soak-in medium is eliminated from the bath before counting during efflux.

The efflux curves obtained in these ways (Figs. 3 to 5) showed differences between right and left auricles like those which occurred in the influx. The left auricles exchanged fairly and uniformly, though with a moderate decline in the rate after the first hour. In the right auricles, a semi-logarithmic plot was more curved, and not readily divisible into separate fractions. The initial rate was always faster than that observed in left auricles, and the final rate after 3 to 4 hr. was slower (Fig. 3).

Calculation of Potassium Fluxes in Normal Auricles.—These results make possible approximate calculations of the potassium fluxes of the auricles. In left auricles it seems reasonable to assume that there is no serious inhomogeneity in the cells, and that the movements decline somewhat during immersion. Most of the decline in rate probably occurs in the first half-hour; and it has not much influence in interpreting efflux experiments in which the measurements of radioactivity are made after the first 90 min. The basis of the calculations has been given by Keynes and Lewis (1951) and Keynes (1954). The rate of

influx is given by the product of initial rate of entry of tracer to the tissue and the sensitivity of the apparatus. The initial rate of entry of tracer can be obtained from the count of the tissue, Y, at time T (for example at the end of a period of influx and beginning of efflux) by the expression

$$\left(\frac{dY}{dt}\right)_{t=0} = \frac{Y}{T}\left(\frac{kT}{1-e^{-kT}}\right) \qquad (1)$$

where k is the rate constant and is given by the observations on the efflux. The sensitivity of the counting apparatus was obtained by ashing the auricle at the end of the experiment and comparing its specific activity with that of the active saline used for influx when each was counted in a liquid counter. The final count in the efflux apparatus then corresponded to the quantity of potassium found to be exchanged at this time, and the uptake at other times could be obtained proportionally. Thus in the experiment shown in Fig. 5 at $T=80$ min. $Y=9140$ counts/min. and $k=-0.0115$ min.$^{-1}$, giving the factor 1.53 for the conversion of Y/T to $(dY/dt)_{t=0}$. This factor is rather large, but it seems preferable not to use counts earlier in influx because of the uncertainty whether the auricle was located in exactly the same position on each transfer to the counting bath. The counting sensitivity was 4.73×10^{-4} micromoles/count, so the average entry to the whole tissue was 0.0955 micromoles/min., corresponding to a flux of 4.43 p.mole. cm.$^{-2}$ sec.$^{-1}$

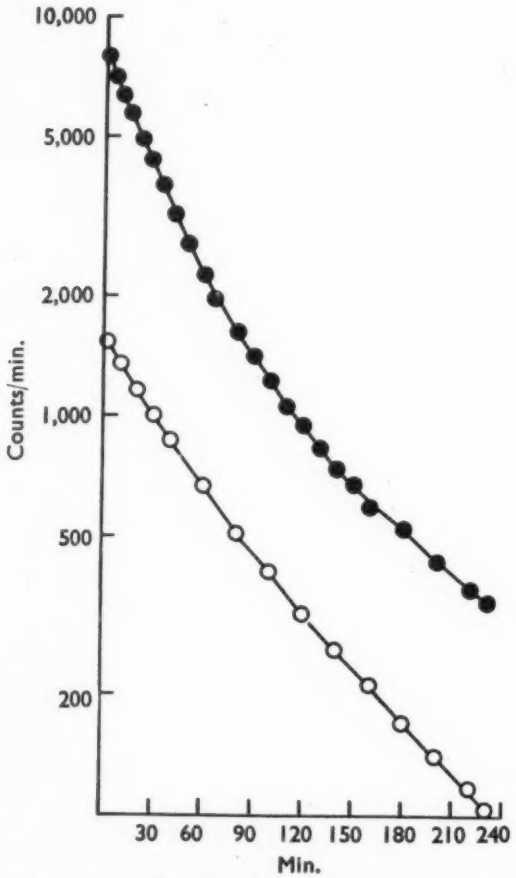

FIG. 3.—The loss of ^{42}K from rabbit auricles, plotted semilogarithmically. ●, Right auricle. ○, Left auricle.

Efflux can be calculated from the expression

$$m_0 = k\,\frac{V}{A}\,C_i \qquad (2)$$

(Keynes and Lewis, 1951), where V is the volume of the tissue cells, C_i the concentration of potassium in them, and A the surface area of the cells. For the whole tissue the outward movement is $k.V.C_i$ which in this experiment gives the value 0.0962 micromoles/min. These figures require correction for diffusion, as discussed below, and they involve such approximations so that the net loss of potassium as estimated by the difference between influx and efflux is very unreliable: however, its value 0.0007 micromoles/min. or about 0.5%/hr. is as near to the average loss of 2%/hr. observed throughout these experiments as can be expected.

The correction for delays due to diffusion has been discussed by Harris and Burn (1949), Keynes (1954) and Creese (1954). All these authors have found apparent diffusion coefficients of potassium in the extracellular spaces to be about one-quarter of those for the ion in free solution, namely of the order of 5×10^{-6} cm.$^{-2}$ sec.$^{-1}$ This value may be used for D' in the expressions of Keynes (1954).

$$\frac{M'}{M} = \frac{\lambda}{b}\tan h\,\frac{b}{\lambda} \qquad (3)$$

and

$$\lambda 2 = \frac{\epsilon}{1-\epsilon}\,\frac{D'V.C_0}{M.A} \qquad (4)$$

where ε is the fraction of the volume of tissue occupied by extracellular fluid, C_0 the concentration of ions in the extracellular fluid (taken as equal to that in the medium), M is the true flux and M' the apparent one, and b is half the thickness of the tissue. In view of the uncertainties attaching to a number of these estimates, it is hardly worth calculating the correction afresh for every separate auricle, and a value of 1.3 has been used for M'/M as representing the median of a number of observations on auricles of approximately average size. The corrected fluxes then

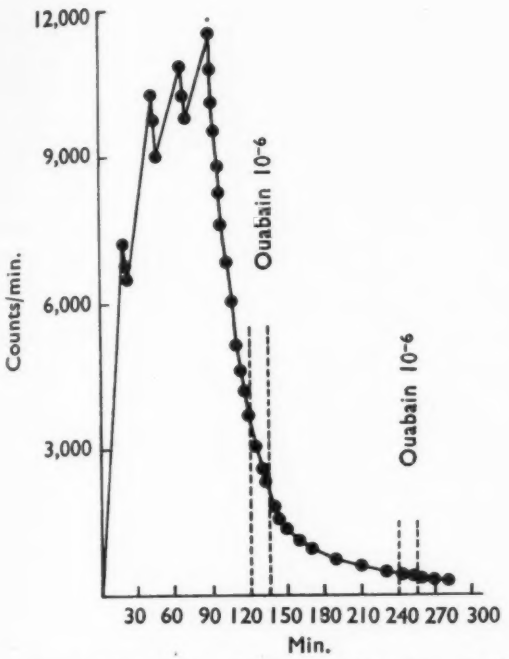

FIG. 4.—The uptake and loss of ^{42}K in a right auricle. Ouabain (10^{-6}) was included in the perfusion medium for two periods of 15 min. during the efflux, at the times shown by the dotted lines.

become 5.75 and 5.81/mole cm.$^{-2}$ sec.$^{-1}$ respectively.

Potassium Fluxes in Normal Right Auricles.—The application of similar arguments to the results with right auricles is hampered by the evident inhomogeneity of the potassium exchange and the presumable fluctuations in the influx and efflux with each contraction. It is, at first sight, unlikely that beating alone accounts for the non-exponential uptake and output of ^{42}K, and the similarity of exchange in left auricles whether they are driven by remaining connected to right ones or are independent and inactive (Table III) confirms this point. It seems more likely that some part of the tissue has a more rapid turnover than the rest: the pacemaker might so function, as it is known to show electrical activity independently of the rest of the auricle (Marshall and Vaughan Williams, 1956). Whether there are two distinct fractions or a gradation of cells exchanging at different rates is not evident from the results: the progressive curvature of the semi-logarithmic plot may indicate such a gradation (Creese, Neil, and Stephenson, 1956), but it could also result if there were two distinct fractions of which the exchange rates were gradually decreasing during the time in which the preparation was studied. In the circumstances it appears un-

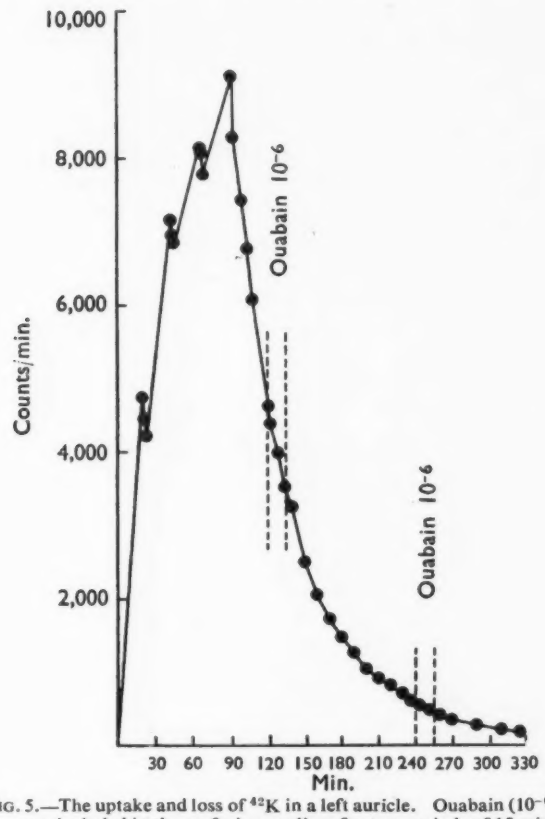

FIG. 5.—The uptake and loss of ^{42}K in a left auricle. Ouabain (10^{-6}) was included in the perfusion medium for two periods of 15 min. during the efflux, at the times shown by the dotted lines.

desirable to attempt a more quantitative interpretation of the results.

Potassium Movements in the First Hour After Immersion.—It is also possible to estimate the influx of potassium in the early part of the time after immersion in saline from observations such as those shown in Fig. 2, by using expression (1) above. Appreciable errors arise if the total exchange of the tissue is less than about a quarter, because the extracellular fluid, which must exchange more rapidly than the bulk of the tissue, then makes an appreciable contribution to the total. Also, in the initial period the rate of efflux is uncertain and so there is some doubt about the appropriate value for k. It is possible that the rate of efflux declined rapidly *pari passu* with the influx: it is also possible that the rate of efflux did not change, and that the initial rapid influx involved a net gain of potassium by the tissue, perhaps recovering losses which occurred during dissection. There was too much variation in the estimated potassium content of individual auricles to distinguish these possibilities, and it seemed reasonable to adopt the simpler assumption that the

TABLE IV
ESTIMATED INFLUX OF POTASSIUM IN RABBIT AURICLES

Treatment	Period of Immersion Before Measuring Uptake (min.)	Duration of Measurement (min.)	Mean Influx During Period (mEq./kg. Dry Wt. of Tissue/min.)	
			Left	Right
Control	0	10	8·3	12·1
Ouabain 10^{-6} ..	0	10	7·7	10·5
,, 4×10^{-6}	0	10	5·7	6·7
,, 10^{-5} ..	0	10	5·1	5·5
Digoxin 10^{-5} ..	0	10	6·1	7·1
Control	20	20	6·3	11·0
Ouabain 10^{-7} ..	20	40	4·4	6·8
,, 10^{-6} ..	20	40	3·3	3·3
,, 10^{-5} ..	20	20	2·2	1·4
Control	90	20	5·0	8·4
Ouabain 10^{-5} ..	90	20	1·6	1·5

efflux was constant at a rate of -0.015 min.$^{-1}$ for left and -0.035 min.$^{-1}$ for right auricles. Even if the true rates were twice this magnitude it would increase the inward movements estimated on the influx for 20 min. by only 10 to 20%, so the error is unlikely greatly to exaggerate those which arise from variation between individual tissues. Some values obtained in this way are shown in Table IV, for auricles immersed in active saline for varying periods either immediately or 20 or 90 min. after dissection. The correction for diffusion varies somewhat for different auricles, because they varied somewhat in size, but in view of the other approximations a uniform correction $\times 1.3$ has been applied as before to all the estimations for this effect.

Effects of Digoxin and Ouabain

Gross Effects.—When digoxin or ouabain was added to the medium in sufficient concentration, right auricles or pairs of auricles initially beat more forcibly and more rapidly. Irregularities both of rate and magnitude appeared, transiently or persistently, and after a time the auricles ceased to beat. Sporadic contractions or periods of contraction sometimes occurred later, especially with digoxin.

Once irregularities had begun, the size of the beat appeared to depend mainly on the previous activity of the tissue, being largest after a quiescent spell: that is, there was a negative staircase effect. The highest concentrations of drug acted more rapidly: as a measure of the effect, the time from exposure to the drug till there was no visible contraction for more than 30 sec. was measured in a number of pairs of auricles (Table V). The results shown there are consistent with a large number of other experiments in which the tissues were not observed continuously and the time to arrest was noted only approximately. The electrical activity of the auricles as a rule showed no augmentation except in frequency: the spikes became lower with little change in the steepness of the rising phase, but considerable flattening of the descending part. Later the form of individual pulses became increasingly irregular. Detectable electrical activity persisted after visible mechanical activity had ceased.

TABLE V
TIME OF ARREST OF BEAT AFTER EXPOSURE TO DIGOXIN AND OUABAIN

Drug	Concentration	Duration of Exposure Causing Arrest (min.)
Digoxin	10^{-6}	>150
	10^{-5}	16, 28, 30, 45
Ouabain	10^{-7}	>150
	10^{-6}	32, 40, 45, 45, 48
	10^{-5}	4,10

Net Changes in Composition.—Loss of potassium occurred consistently from auricles immersed in media containing digoxin or ouabain (Fig. 6).

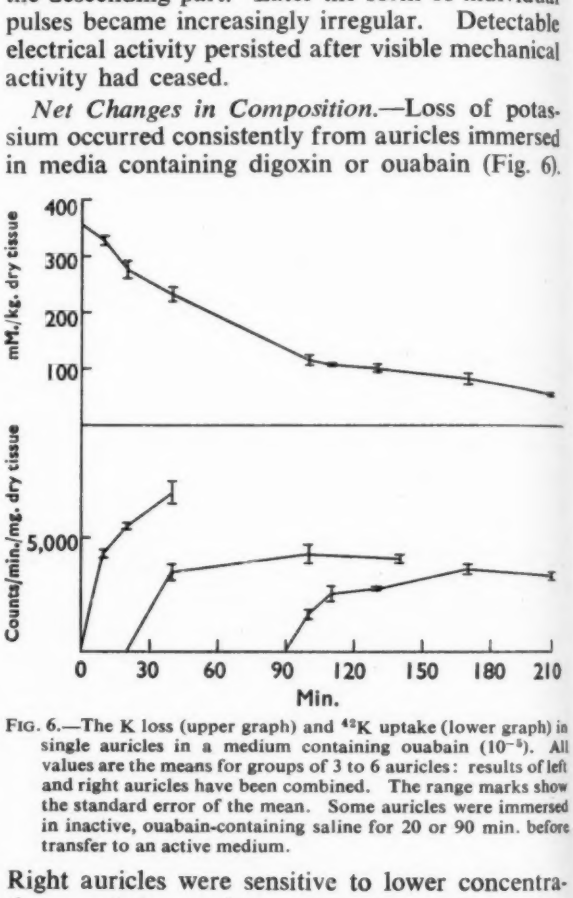

FIG. 6.—The K loss (upper graph) and ^{42}K uptake (lower graph) in single auricles in a medium containing ouabain (10^{-5}). All values are the means for groups of 3 to 6 auricles: results of left and right auricles have been combined. The range marks show the standard error of the mean. Some auricles were immersed in inactive, ouabain-containing saline for 20 or 90 min. before transfer to an active medium.

Right auricles were sensitive to lower concentrations and in a given concentration lost more rapidly than left auricles. On both sides, the rate of loss of potassium was greater in higher concentrations of ouabain or digoxin. In all the conditions examined, the mean amount of potassium in the right auricle had fallen to about 280 mEq./kg. dry weight at the time when beating ceased, as defined above. There were no significant changes

in the sodium and water content of the auricles except in 10^{-5} ouabain in which there was a considerable gain of sodium. However, it must be remembered that the large and moderately variable extracellular volume makes the total tissue sodium a poor indication of the quantity present in the cells, and the observations do not exclude an increase of intracellular sodium comparable in magnitude to the loss of potassium in 10^{-6} ouabain and 10^{-5} digoxin.

^{42}K Influx.—The loss of potassium was accompanied by a reduced entry of ^{42}K to the auricles (Fig. 6). Ouabain was again more active than digoxin in the same concentration, and right auricles were more sensitive than left. Thus, the rapid component of the uptake of right auricles was practically abolished by 10^{-5} digoxin, which had only a small and delayed effect on the left, and 10^{-6} ouabain reduced the uptake on the right without significant effect on the left. These effects were observed when the auricles were exposed to the cardiac glycoside and ^{42}K simultaneously, and since some time is necessary for the drug to reach and act on the cells which it affects, they are not maximal effects. The rate of entry of ^{42}K was further reduced in auricles which had been in saline containing 10^{-5} ouabain for 20 min. before transfer to an active saline with the same concentration of ouabain (Fig. 6), and with 90 min. initial exposure the rate of entry was still lower. The auricles by this time had lost three-quarters of their total potassium, had gained much sodium, and had been inactive probably for over 80 min., so many factors may have affected the result. In these conditions the potassium remaining in the auricles was still completely exchangeable, and somewhat more rapidly (within 160 min.) than in normal conditions.

^{42}K Efflux.—The simplest observations were made by applying media containing ouabain or digoxin for periods of 15 min. to auricles while ^{42}K efflux was being followed, and then reverting to a normal perfusion medium. In these conditions there was no detectable effect from ouabain (10^{-6}) either on the rapid or the slow phases of efflux from right auricles (Fig. 4), although gross mechanical and electrical changes occurred during each exposure and were reversed on restoring a normal medium.

Somewhat different results, which at first suggested a decreased rate of efflux, were obtained in experiments in which pairs of auricles were immersed in active saline containing 10^{-5} digoxin for 30 min. and then allowed to lose their ^{42}K in an inactive medium, still containing digoxin. Usually, two such sequences were performed with the same pair of auricles, once with and once without digoxin, and sometimes the digoxin was used in the first cycle and sometimes in the second. These experiments were performed in conditions commonly used for studying this tissue, namely contracting isotonically in a conventional organ bath at 28°, and loss of radioactivity was followed by removing the medium for counting and replacing it every 5 or 10 min. The results gave an average value for the loss from the whole tissue. After the first half hour, the rate of loss of ^{42}K declined steadily, with a half time of 50 min. for normal auricles and 64 min. for those in 10^{-5} digoxin. In the light of the observations on influx in left and right auricles separately, it is probable that in the presence of digoxin less ^{42}K had entered the rapidly exchanging fraction of the right auricle and the apparently slower rate of efflux was due to the absence of a rapid component and not to a general reduction in the rate of efflux.

Calculation of the Rates of Movement in the Presence of Ouabain.—The rates of outward movement of potassium in the presence of ouabain can be calculated either by assuming that there is no change in efflux at any time and using the observed efflux rate and the observed net loss of potassium: or by using the observed influx of tracer and assuming that the change in the efflux is not large enough seriously to alter the value of $kT/(1-e^{-kT})$. The results of the two methods agreed in all the conditions examined to within about 25%, which is as good as the various approximations used are likely to allow: and this degree of agreement between independent measurements gives some confidence that their magnitude is correct. As shown in Table IV, the cardiac glycosides exert a larger effect when they are allowed to act for some time, though this is complicated by the decline in the rate of potassium influx which occurs in control auricles. With a sufficiently high concentration of ouabain (10^{-5}) the influx is reduced by one-third even in the first 10 min. and, as noted above, this results in a loss of about one-tenth of the tissue potassium and cessation of the beat. With lower concentrations, the efflux is reduced less rapidly, and it is possible that the failure to observe any effect from concentrations of 10^{-7} or lower is a result of not prolonging the experiments sufficiently.

Discussion

The measurements of potassium movements made on control auricles are of limited significance because they apply to non-beating tissue over

relatively long periods and do not give information about the movement which occurs at each systole and diastole. In tissues of the dimensions of rabbit auricles, it is likely that much of the potassium presumably extruded at each beat (Wilde, O'Brien and Bay, 1955) is promptly returned from the extracellular fluid and only part is involved in the observed exchanges of the tissue as a whole. This is borne out by observations on left auricles, where the influx rate was little affected whether the tissue was separated from the pacemaker in the right auricle, or was driven by it while exchange was measured. The observation that part of the potassium of the right auricles exchanges at a fairly similar rate is also suggestive. If beating *per se* substantially altered the exchange rate of the tissue, it ought to do it for the whole tissue, and not to account for acceleration during only part of the exchange. Indeed, the fact that such tracer as remains in the auricles after more than 2 hr. of efflux exchanges more slowly in the right auricle suggests that beating may diminish the overall exchange of the tissue, by promoting reabsorption of extruded ^{42}K instead of letting it escape from extracellular fluid. However, other factors may also be involved in left-right differences.

The significance of the rapid part of the exchange in right auricles is uncertain, and the evidence is insufficient to distinguish between a rapid fraction in all the cells, and the separate existence of a group of cells with a fast exchange, or a gradation of exchange rates in different cells. It is tempting to associate the faster exchange with the pacemaker. The amount of tissue involved has not been estimated from the present results; it looks as though it is larger than the anatomically defined pacemaker (Keith and Flack, 1907), but wider areas may show spontaneous firing (Marshall and Vaughan Williams, 1956), and so could account for the rapid component. It evidently requires further investigation. Whatever its identity, it is important in understanding the action of cardiac glycosides, since it is more sensitive to their action than the rest of the tissue: this is quite consistent with its identity as a pacemaker.

It may be noted that Schreiber (1956) has described two phases of potassium exchange in frog hearts and finds only the slow fraction sensitive to ouabain. This is at variance with the present observations, but there are considerable differences in conditions and insufficient evidence to relate his components to those described here.

As has been observed before (Hajdu, 1953; Conn, 1956; Schreiber, 1956), cardiac glycosides plainly inhibit the influx of potassium with little or no effect on efflux unless or until there is a considerable reduction in the intracellular concentration of potassium. Their effect on sodium movement has not been examined: potassium loss is accompanied by sodium gain, but whether this is due to decreased extrusion or increased influx is not established. Numerous observations on other tissues suggest that the former is more likely (Matchett and Johnson, 1954).

It seems probable that the loss of potassium from the tissue is connected with some of the effects produced by the glycosides. The failure of contraction in different concentrations of digoxin or ouabain occurred when the intracellular potassium had fallen by about 15%, and it is well known that raising the external potassium concentration delays the onset of digitalis-induced failure. It would be dangerous to try to deduce what changes in membrane potential are occurring, since the loss of potassium from the cells, at least with high concentrations of ouabain, is sufficiently rapid to be likely to increase locally the concentration of potassium in the extracellular fluid and, in any case, the drug may modify the electrical properties of the membrane in quite unknown ways. It is possible that the intermittent beating which occurs in concentrations of digoxin or ouabain which reduce the tissue potassium to but not beyond the critical level occurs in this way. It is likely that most of the potassium efflux is associated with each beat, and that, once beating ceases, the efflux from the cells is reduced. With the inward, active transport impaired but not abolished, this reduction in efflux may well be sufficient for the tissue to regain a little potassium and so restore conditions in which beating is possible. Extrusion at each beat then progressively lowers the internal concentration again, and the characteristic negative staircase and further stoppage follow. The fact that it is the mechanical response which diminishes progressively, and that electrical activity persists when beating has ceased, suggests that disturbance of the membrane potential is not the principal element in failure.

It is somewhat surprising that the concentrations of ouabain and digoxin necessary to produce these effects are much higher than those likely to occur in therapeutic conditions, and also than those which produce detectable effects in erythrocytes (Kahn and Acheson, 1955; Joyce and Weatherall, 1955; Glynn, 1955). The observed results suggested that lower concentrations might be effective if they were allowed to act for longer periods; here no such effects were followed for more than about 2 hr. However, it must be

remembered that other components of the mechanism of the heart, such as isolated actomyosin, are sensitive to the action of ouabain (Robb and Mallow, 1953) and little is yet known about the way in which these various components affect each other in the work of the whole heart.

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References

Bacon, J. S. D., and Bell, D. J. (1948). *Biochem. J.*, **42**, 397.

Baker, J. B. E. (1947). *Brit. J. Pharmacol.*, **2**, 259.

Calhoun, J. A., and Harrison, T. R. (1931). *J. clin. Invest.*, **10**, 139.

Clark, A. J. (1912). *Proc. R. Soc. Med.*, **5**, 181.

Conn, H. L. (1956). *Amer. J. Physiol.*, **184**, 548.

Creese, R. (1954). *Proc. R. Soc., B*, **142**, 497.

—— Neil, M. W., and Stephenson, G. (1956). *Trans. Faraday Soc.*, **52**, 1022.

Friedman, M., and Bine, J. (1947). *Amer. J. med. Sci.*, **214**, 633.

Glynn, I. M. (1955). *J. Physiol.*, **128**, 56*P*.

Hajdu, S. (1953). *Amer. J. Physiol.*, **174**, 371.

Harris, E. J., and Burn, G. P. (1949). *Trans. Faraday Soc.*, **45**, 508.

Hazard, R., Hazard, J., and Thouvenot, J. (1956). *Arch. int. Pharmacodyn.*, **105**, 33.

Joyce, C. R. B., and Weatherall, M. (1955). *J. Physiol.*, **127**, 33*P*.

Kahn, J. B., Jr., and Acheson, G. H. (1955). *J. Pharmacol.*, **115**, 305.

Keith, A., and Flack, M. (1907). *J. Anat., Lond.*, **41**, 172.

Keynes, R. D., and Lewis, P. R. (1951). *J. Physiol.*, **113**, 73.

—— (1954). *Proc. R. Soc., B*, **142**, 359.

Lown, B., Salzberg, H., Enselberg, C. D., and Weston, R. E. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 797.

Marshall, J. M., and Vaughan Williams, E. M. (1956). *J. Physiol.*, **131**, 186.

Matchett, P. A., and Johnson, J. A. (1954). *Fed Proc.*, **13**, 384.

Miles, A. E. W., and Linder, J. E. (1952). *J.R. micr. Soc.*, **72**, 199.

Regan, T. J., Talmers, F. N., and Hellems, H. V. (1956). *J. clin. Invest.*, **35**, 1220.

Robb, J. S., and Mallow, S. (1953). *J. Pharmacol.*, **108**, 251.

Schatzmann, H.-J. (1953). *Helv. physiol. acta*, **11**, 346.

Schreiber, S. S. (1956). *Amer. J. Physiol.*, **185**, 337.

Wedd, A. M. (1939). *J. Pharmacol.*, **65**, 268.

Wilde, W. S., O'Brien, J. M., and Bay, I. (1955). *Circulation*, **12**, 788.

Wood, E. H., and Moe, G. K. (1938). *Amer. J. Physiol.*, **123**, 219.

Brit. J. Pharmacol. (1957), **12**, 382.

THE ASSOCIATION OF BLOOD CHOLINESTERASE LEVELS WITH THE SUSCEPTIBILITY OF ANIMALS TO SARIN AND ETHYL PYROPHOSPHATE POISONING

BY

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(RECEIVED MAY 29, 1957)

An association between the blood cholinesterase (ChE) levels and the toxicity of sarin and ethyl pyrophosphate has been established. This has been demonstrated in two types of experiment. In the first, guinea-pigs were given a non-lethal dose of sarin (*iso*propyl methylphosphonofluoridate) which reduced the blood ChE to 20% of normal, and at intervals, as the ChE level of the blood gradually recovered, separate batches were given a second dose of the same size. A comparison was then made between the blood ChE levels immediately prior to the injection of the second dose and the mortality rate. In the second, a relatively small dose of sarin or ethyl pyrophosphate was given daily to rabbits until the enzyme value fell to a steady level. LD50 values were then determined on such groups and compared with those found in saline treated ones. In both guinea-pigs and rabbits it has been shown that a depression of blood ChE to below 40% indicated an increased toxicity of sarin and ethyl pyrophosphate to these species. The approximate quantitative relation appears to be that susceptibility is increased in the ratio 1.5 when the blood ChE is reduced by half.

Toxic manifestations in anticholinesterase poisoning are directly related to the inhibition of the enzyme in the central nervous system, the parasympathetic nervous system and the neuromuscular junction (Koelle and Gilman, 1949). The level of cholinesterase (ChE) activity in the blood gives no indication of the extent of inhibition at these sites, consequently there is no close relationship between the variation of blood ChE and the onset of symptoms. Thus it is difficult to assess the precise significance of blood ChE levels in anti-ChE poisoning. Marchand (1952), however, has stated that ChE testing of the red cells and plasma appears to be a specific and reliable method of establishing or excluding significant intoxication by organo-phosphorus insecticides or war gases.

Davies (1952) pointed out that ChE estimations have been of diagnostic value in individual cases of poisoning in which the symptoms have been ambiguous and he also drew attention to the importance of low ChE values as an objective index of inadequate safety precautions or faulty handling of such compounds.

Fawley, Hagen and Fitzhugh (1952) showed that while plasma and erythrocyte ChE measurements appear to be of value in confirming a suspected acute exposure to anti-ChE compounds, they offer little help in evaluating the degree of exposure and severity of symptoms. In chronic exposures, however, ChE measurements may be of considerable value.

One aspect of the importance of blood ChE levels has not been adequately investigated. The organo-phosphorus insecticides inactivate the enzyme through the formation of a stable phosphorylated ChE, which varies with the compound (Burgen, 1949; Aldridge, 1954). The return of ChE activity to both red cells and plasma is thus very slow and it is therefore of considerable importance to determine whether the low level of the blood enzyme under these conditions is indicative of a state of increased susceptibility to subsequent doses of anti-ChE.

This paper reports the results of experiments in which the blood ChE level of mammals, lowered either by sarin (*iso*propyl methylphosphonofluoridate) or ethyl pyrophosphate, was related to

susceptibility to further treatment with the anticholinesterase used. In both guinea-pigs and rabbits we have found that a depression of blood ChE activity to below 40% was followed by an increased toxicity of sarin and ethyl pyrophosphate to these species.

Materials and Methods

Guinea-pigs and rabbits were used, but since the experiments were carried out over a long period (12 to 18 months) no rigid standardization of strain or sex was possible although all experiments were performed on adult animals.

Two anti-ChE agents were used, sarin and ethyl pyrophosphate. Both were prepared in this establishment and were more than 95% pure.

Guinea-pigs were injected subcutaneously and rabbits intravenously.

ChE determinations were carried out upon whole blood. Two methods of analysis were employed. In the earlier experiments, the Warburg manometric technique (Callaway, Davies, and Rutland, 1951) was used, but in later work the convenience of the electrometric method (Michel, 1949) made it the procedure of choice. Acetylcholine was used throughout as the substrate.

The unit of enzyme activity varied with the method of analysis used and the species under investigation, but since they were kept constant in any given experiment and the results were compared only with those obtained in the same experiment, these differences in units were unimportant. For clarity, however, the units of activity are defined in the results section appropriate to the experiment.

Results

Guinea-pigs

In the first, a batch of 100 animals was taken and from this eight were randomly chosen for the evaluation of their normal whole blood ChE. Each guinea-pig in the batch of 100 was then injected with 0.03 mg./kg. sarin, a dose which preliminary experiments had shown would produce a substantial reduction in ChE level but little or no mortality. In fact, no guinea-pigs died at this dose level and blood taken from the eight animals used for determination of pre-exposure ChE levels showed an average reduction to 23% of normal. These eight, together with an additional four, were given 0.03 mg./kg. sarin two hours after the first injection. All 12 died within a few minutes and blood obtained by heart puncture from the original eight showed that the ChE level had been reduced to an average of 5% of normal.

Twenty-four hours later a further 12 animals from the original batch were selected and blood samples taken. They were injected immediately afterwards with another dose of 0.03 mg/kg. sarin. Two hours later, or immediately after death, blood samples were again taken. A similar procedure was adopted with subsequent batches of 12 animals on the third, fourth, fifth, eighth, tenth and eleventh days. Thus for each of these batches, ChE activities were obtained before and after the second injection together with mortality records.

This experiment was repeated about one year later using 42 guinea-pigs. Batches of 6 were taken successively, the doses of sarin being the same as in the first series.

Table I

THE RELATION BETWEEN BLOOD CHOLINESTERASE ACTIVITY LEVEL AND MORTALITY IN GUINEA-PIGS

The dose of sarin was 0.03 mg./kg. given in a 1/1,000 dilution of saline. The activity, measured by the Warburg manometric method, is expressed as % of the mean pre-exposure value.

Day	Expt. 1			Expt. 2		
	Mean ChE Activity		Mortality	Mean ChE Activity		Mortality
	Before Injection	After Injection		Before Injection	After Injection	
0 ..	100	23	0/100	100	16	0/42
2 hr. ..	23	5	12/12	16	7	5/6
1 day ..	36	9	8/10	37	6	6/6
2 days ..	42	13	4/12	41	8	2/6
3 ,, ..	56	21	1/11	47	12	1/6
4 ,, ..	61	11	1/12	58	10	0/6
7 ,, ..	73	14	2/12	73	18	0/6
9 ,, ..	73	16	0/12			
10 ,, ..	78	19	0/12	78	21	0/6

In both experiments (Table I) the initial dose of sarin reduced the blood ChE activity to about 20% of its normal value without producing any mortality. Two hours later a second dose of the same size reduced it to about 6% of normal and killed a total of 17 out of 18 animals in the combined experiments. Regeneration of the blood ChE activity occurred exponentially, rising to 78% of normal 10 days after the initial dose. Thus each successive batch of guinea-pigs was injected with a constant dose (0.03 mg./kg.) of sarin at a mean ChE level higher than that of the preceding batch.

The mortality produced by this dose fell with the rising ChE activity and from Table I it may be deduced that 0.03 mg./kg. sarin would have killed only half of the guinea-pigs between the first and second day after the initial dose, when the mean ChE level was about 40% of its normal value.

TABLE II

THE MEAN % REDUCTION IN BLOOD CHOLINESTERASE ACTIVITY IN GUINEA-PIGS PRODUCED BY A DOSE OF SARIN

The dose of sarin was 0.03 mg./kg. The units of ChE are expressed as $\mu l.CO_2$/30 min./0.1 ml. of whole blood. Substrate, 0.006 M acetylcholine. Method, Warburg manometric. These results were obtained from Experiment 1, Table I.

Initial ChE (Units)	Mean % Reduction	S.E. of Mean	No. of Observations
20– 39	77	3·5	11
40– 59	71	3·1	15
60– 79	69	2·9	16
80– 99	77	1·1	32
100–119	78	1·2	10
120–139	80	2·7	6
140–159	76	2·9	3
Mean % reduction	74·9		

Another point which emerged was that the % reduction of ChE activity was practically independent of the initial ChE level. If the ChE levels, taken immediately before the second injection, are grouped, the % fall produced by 0.03 mg./kg. sarin appears reasonably constant (approximately 75%) though fairly large individual variations occur. This is shown for the first experiment in Table II.

Rabbits

In the foregoing experiments on guinea-pigs, the dose of sarin was kept constant and its effects at various levels of blood ChE were studied and these levels were inseparably associated with varying times after the injection of sarin. A different approach was adopted in the experiments with rabbits in that different doses of sarin were given to rabbits with a lowered blood ChE activity produced by daily injections of one-quarter of the LD50 of sarin.

In a subsequent experiment ethyl pyrophospate was used instead of sarin.

Sarin.—Sixty rabbits were randomized in six groups of 10. Blood, taken from the marginal ear vein of each rabbit, was pooled for each of the six groups. Each ChE determination thus provided an estimate of the mean level of activity for each group of 10 rabbits. Three of the groups then received daily intravenous injections of 0.005 mg./kg. sarin given as 1/10,000 dilution in saline. These repeated sublethal doses of sarin produced marked ChE inhibition without the accompaniment of serious signs or symptoms of sarin poisoning.

The remaining three groups were used as controls and received equivalent injections of saline (0.05 ml./kg.) daily.

One rabbit in each of the sarin treated groups died during the course of the injections; one after the fourth and two after the fifth. One rabbit in the saline series was in a very weak condition and was excluded from the toxicity determinations. The remaining rabbits were in good condition and showed no signs of sarin poisoning.

On the day following the sixth injection, all the surviving animals were again bled and the six pooled samples of blood used for the determination of ChE activity. On the second day after the sixth injection, the LD50 for sarin was determined for each group. Cholinesterase levels in the poisoned and control groups, both before and after the completion of injections, are shown in Table III for two separate but similar experiments.

TABLE III

WHOLE BLOOD CHOLINESTERASE LEVELS IN RABBITS BEFORE AND AFTER SIX INJECTIONS OF SALINE OR OF SARIN

ChE activity, determined by the Michel electrometric method on pooled blood from 10 rabbits in each group, is expressed as ΔpH-90 min./0·5 ml. whole blood × 100. The dose of sarin used was 0·005 mg./kg. Substrate, 0·015 M acetylcholine. For a further explanation of the results see text.

Group	Treatment	Whole Blood ChE Activity				% Inhibition	
		Expt. 1		Expt. 2			
		Before	After	Before	After	Expt. 1	Expt. 2
A	Sarin	169	53	131	61	69	53
B	,,	144	49	139	75	66	46
C	,,	139	54	140	63	61	55
D	Saline	143	120	143	117	16	18
E	,,	150	127	138	112	15	19
F	,,	151	131	137	115	13	16

The degree of inhibition in each poisoned group agreed closely, the mean inhibitions being 66% and 51%. In each experiment there was a small but definite reduction in the saline treated groups, the cause of which is unknown. The values given below for the net inhibition due to sarin have therefore been calculated from the expression $100(I_p - I_s)/(100 - I_s)$, where I_p and I_s are the observed % inhibitions due to anticholinesterase and saline respectively.

A six point assay of the intravenous toxicity of sarin in the poisoned and control groups was carried out. Fig. 1 shows the dosage/mortality regression lines on log probability co-ordinates for the two experiments. These lines were calculated by Probit analysis (Finney, 1952) using several cycles of iteration. In each case there was no significant departure from parallelism between the control and poisoned groups.

In the first experiment, the values for the intravenous LD50 of sarin for the treated and control groups were 0.013 mg./kg. and 0.020 mg./kg. respectively. The relative toxicity of sarin in the

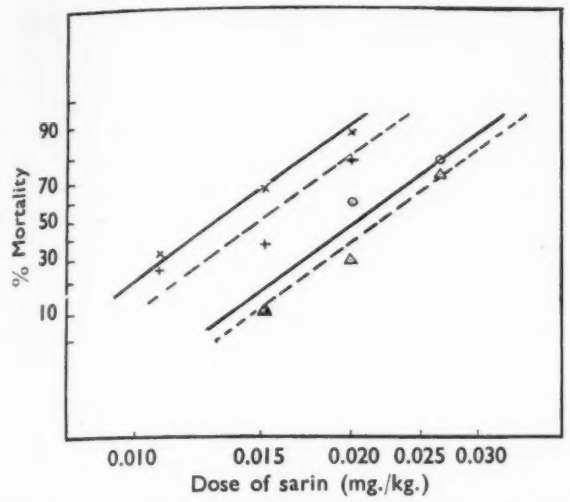

FIG. 1.—Dosage/mortality regression lines for saline and sarin treated rabbits. Expt. 1, X——X sarin treated group; ⊙——⊙ control saline group. Expt. 2, +– – –+ sarin treated group; △– – –△ control saline group.

two groups was thus 1.55 (1.22 to 2.11, $P=0.95$) for a net % ChE inhibition of 60%. The corresponding values for the second experiment were: LD50, treated 0.016 mg./kg., control 0.023 mg./kg., relative toxicity 1.45 (1.09 to 2.07) and net % ChE inhibition 41%. Thus the susceptibility of rabbits to sarin is increased by about 50% when their blood ChE activity is reduced to a half.

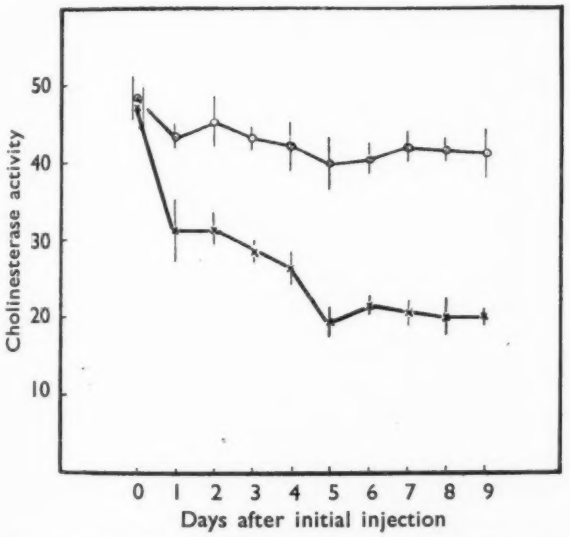

FIG. 2.—Variation in whole blood ChE activity in rabbits receiving daily injections of ethyl pyrophosphate (0.05 mg./kg.) or saline (0.5 mg./kg.). Each point represents the mean value of 6 groups in which blood from 5 rabbits was pooled. The vertical lines indicate the standard deviation. ⊙——⊙, saline group; X——X, ethyl pyrophosphate group.

Ethyl Pyrophosphate.—In these experiments ethyl pyrophosphate (TEPP) was used instead of sarin. Sixty rabbits were randomized, this time in twelve groups of 5, and venous blood from each rabbit was pooled for each of the 12 groups. Six of the groups received daily intravenous injections of 0.05 mg./kg. of TEPP in saline (0.1 mg./ml.). The remaining six groups received daily injections of saline (0.5 ml./kg.).

One rabbit in the TEPP treated group died during the conditioning period and reference to it was excluded from the records. The remaining animals showed no cholinergic symptoms and were in good condition.

The injections of TEPP produced a progressive fall in blood ChE activity up to the fifth day, after which it remained steady at about 40% (Fig. 2).

On the day following the ninth injection final determinations of the ChE activity were made and the LD50 for TEPP was determined for each of the two groups. At this stage the ChE inhibition of the TEPP injected animals was 57.5%. Saline injection again produced a reduction in ChE activity (13.9%) which was comparable with that found in the sarin experiments (Table IV).

TABLE IV

WHOLE BLOOD CHOLINESTERASE LEVELS IN RABBITS BEFORE AND AFTER SIX INJECTIONS OF SALINE OR ETHYL PYROPHOSPHATE (0.05 MG./KG.), AND INTRAVENOUS LD50 OF ETHYL PYROSPHOSPHATE FOLLOWING THE FINAL INJECTION

The units of ChE activity, expressed as the mean ±S.D. of 6 groups, each comprising pooled blood from 5 rabbits, are $\triangle p$H/hr. × 100, determined by the Michel method using 0·2 ml. blood with 0·015 M ACh as substrate.

Injection of	Whole Blood ChE Activity		% Inhibition	i.v. LD50 Ethyl Pyrophosphate (mg./kg.)
	Before	After 9th Injection		
Ethyl pyrophosphate	47·0±2·5	20·0±0·9	57·5	0·305 (0·243 to 0·382)
Saline ..	48·2±2·7	41·5±3·3	13·9	0·428 (0·400 to 0·456)

Fig. 3 shows the dosage/mortality regression lines on log probability co-ordinates for the final TEPP assay. As for sarin, these lines were calculated by probit analysis using several cycles of iteration. Unlike the sarin regression lines, however, those for TEPP were significantly non-parallel. As a result of this lack of parallelism independent regression lines were calculated and the toxicity ratio between control and poisoned groups obtained for the LD50 only. This toxicity ratio, LD50 Control Group/LD50 TEPP Group, was 1.40 (1.11 to 1.77).

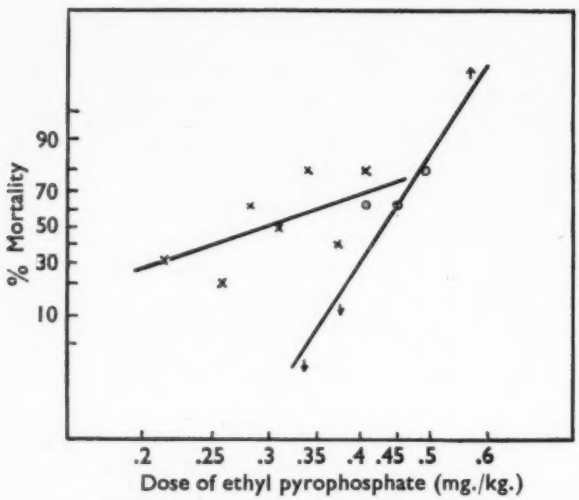

FIG. 3.—Dosage/mortality regression lines for saline and ethyl pyrophosphate treated rabbits X———X ethyl pyrophosphate treated group. ⊙———⊙ controls saline treated.

DISCUSSION

The experiments described above show that the toxicity of sarin or TEPP to the guinea-pig and the rabbit is increased when the ChE activity of the whole blood is markedly reduced below normal by prior treatment with the respective agents. These results would imply therefore that an increased susceptibility to an anti-ChE may be developed by previous poisoning with doses which have shown no clinical signs or symptoms and that the level of the blood ChE is an index of this susceptibility.

In guinea-pigs, the blood ChE was reduced by a single sublethal dose of sarin, and the regeneration of ChE activity was used to provide different enzyme levels. It was found that when the blood ChE returned to about 40% of normal, the same sublethal dose of sarin (equivalent to about 70% of the normal LD50) had become the LD50 dose.

In the rabbit experiments where the ChE activity was reduced by daily injections of sarin or TEPP, a similar result was obtained. Thus, in the first sarin experiment, when the ChE was reduced to 40% the LD50 was reduced to 65% of normal; in the second, a reduction of ChE to 59% was accompanied by a fall in the LD50 to 70%. TEPP produced a reduction of 50% in ChE corresponding with an LD50 which was 72% of normal.

Thus, the approximate quantitative relationship throughout appears to be that susceptibility is increased in the ratio 1.5 when the blood ChE activity is reduced to half. All these results agree very closely when considered in relation to the reduction in LD50 which may be associated with a given reduction in blood ChE activity. However, they have somewhat fortuitously centred around a fixed value for the ChE fall. If, however, the guinea-pig experiments are considered, it may be seen from Table I that there is, in fact, a very close correlation between mortality and ChE level over the range 20% to 80% of normal.

It should be emphasized that the level of blood ChE is no indication of the level in other tissues, and is only an empirical indication of a general state of increased sensitivity of the whole animal towards anti-ChE. In all the experiments described, ChE levels were determined upon whole blood, namely upon a mixture of the true- and pseudo-ChE, and it is by the use of whole blood analyses that the association between reduced ChE activity and increased susceptibility has been established. This is a matter of some practical importance, since it is much more convenient to determine ChE activity in whole blood than in separated cells or plasma.

One final point remains to be discussed, that is the difference between the slopes of the regression lines in the TEPP experiment compared with the marked parallelism in the sarin experiment. Differences in slope between dosage mortality regression lines frequently reflect differences in the mode of action of the toxic agents. In our experiments it would appear that the toxic effects of sarin are the same, whether the animal has been pre-treated with sublethal doses of sarin or not. Pre-treatment with TEPP, however, may have modified the mode of toxic action of subsequent doses of TEPP in such a way as significantly to have changed the slope. This difference might be related to differences in stability between the phosphorylated enzymes (Hobbiger, 1956).

The quantitative aspects of the similarity between sarin and TEPP in the increased susceptibility following pre-treatment have been derived from comparison at the LD50. If, however, the differences in slope are taken into account, as may be seen from Fig. 3, animals with lowered blood ChE activity following daily TEPP injections may be even more susceptible to low doses of this agent, namely half the normal LD50 (0.2 mg./kg.), than are the corresponding sarin treated animals, while at doses above the LD50 there may be a corresponding relative increase in the comparative survival rate. Finally, although there are differences in detail between the TEPP and sarin effects, the general conclusion remains, that an increase

in sensitivity to poisoning by either of these agents of the normal LD50) had become the LD50 dose. occurs when they are used to lower the blood ChE activity by repeated injections of sublethal quantities. In these experiments, the lowering of blood ChE was produced by the same agent as that with which the toxicity assessment was carried out. A problem requiring further investigation is whether a similar relationship exists, when the lowering of enzyme is produced by one agent and the final poisoning by another.

References

Aldridge, W. N. (1954). *Chem. & Ind.*, 473.

Burgen, A. V. (1949). *Brit. J. Pharmacol.*, **4**, 219.

Callaway, S., Davies, D. R., and Rutland, J. P. (1951). *Brit. med. J.*, **2**, 812.

Davies, D. R. (1952). *Proc. R. Soc. Med.*, **45**, 570.

Fawley, J. P., Hagen, E. C., and Fitzhugh, O. G. (1952). *J. Pharmacol.*, **105**, 156.

Finney, D. J. (1952). *Probit Analysis*, 2nd ed. Cambridge University Press.

Hobbiger, F. (1956). *Brit. J. Pharmacol.*, **11**, 295.

Koelle, G. B., and Gilman, A. (1949). *J. Pharmacol.*, **95**, 166.

Marchand, J. F. (1952). *J. Amer. med. Ass.*, **149**, 738.

Michel, H. O. (1949). *J. Lab. clin. Med.*, **34**, 1564.

Brit. J. Pharmacol. (1957), **12**, 388.

THE MECHANISM OF ACTION OF MUREXINE ON NEUROMUSCULAR TRANSMISSION IN THE FROG

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Murexine (urocanoylcholine, [2-β-imidazol-4(5)-ylacryloyloxyethyl]trimethylammonium chloride hydrochloride) produced a contracture like acetylcholine in the frog rectus and a neuromuscular block in the rat diaphragm which was not relieved by neostigmine but was antagonized by hexamethonium. Using the foot muscle of the frog, electrical recordings showed that murexine produced a neuromuscular block and depolarized the end-plate region. These effects were similar to those seen with suxamethonium, decamethonium and acetylcholine. While murexine had the same depolarizing potency as decamethonium, it was only one-tenth as active as suxamethonium and acetylcholine. It was concluded that murexine could be classified as a " depolarizing type " of neuromuscular blocking agent but was less potent than suxamethonium.

Murexine (urocanoylcholine, [2-β-imidazol-4(5)-ylacryloyloxyethyl]trimethylammonium chloride hydrochloride) is a naturally occurring choline ester present in high concentration in the hypobranchial body of *Murex trunculus* and other prosobranchiate molluscs. It was first isolated by Erspamer (1948). In a study of the pharmacological actions of murexine, Erspamer and Glässer (1957) reported that it appeared to produce a " depolarizing type " of block in vertebrate neuromuscular transmission. The present paper gives an account of some experiments in which the neuromuscular blocking action of murexine on skeletal muscle has been studied both by pharmacological and by electrical methods.

METHODS

Isolated Frog Rectus Abdominis Muscle.—The muscle was excised from *Rana temporaria* and set up in a 2 ml. bath of normal frog Ringer solution bubbled with air. The contractures were recorded isotonically by a weighted gimbal-lever.

Isolated Rat Phenic Nerve-Diaphragm Preparation.—The method of Bülbring (1946) was modified by using Krebs solution bubbled with 5% CO_2+95% O_2. Single maximal shocks were delivered alternately to the nerve and muscle (Bell, 1957).

Isolated Extensor Longus Digiti IV Muscle *of the Frog.*—The foot muscle was carefully dissected with its nerve and soaked for 30 min. before beginning each experiment in frog Ringer solution containing 1×10^{-6} neostigmine, which was used for all foot muscle experiments. Muscle action potentials, end-plate potentials, and the depolarizing action of drugs (utilizing the moving fluid electrode technique) were recorded using a D.C. amplifier in the manner described by Nicholls and Quilliam (1956).

RESULTS

Frog Rectus.—When added to the fluid bathing the rectus muscle, murexine produced a contracture which developed more slowly than that following a dose of acetylcholine producing a comparable response (Fig. 1). Murexine was about eight times less potent than acetylcholine. The dose/response relations for acetylcholine (solid circles) and for murexine (open triangles) are illustrated in Fig. 2. The slopes of the two curves are approximately parallel, but the curve for murexine is displaced to the right of that for acetylcholine.

Rat Diaphragm.—Murexine produced a neuromuscular block when added to the fluid bathing the diaphragm. This block was not antagonized by neostigmine but was partly reversed by hexamethonium (Fig. 3). The addition of murexine was followed in the experiment illustrated in Fig. 3 by a transient augmentation of the muscle twitch in response to nerve stimulation before neuromuscular block developed. This increased response was seen only once in 12 experiments, whereas Erspamer and Glässer (1957) reported that they often observed a short-lived increase in twitch tension after murexine which preceded neuromuscular block in their cat sciatic-nerve gastrocnemius preparations. Murexine was about

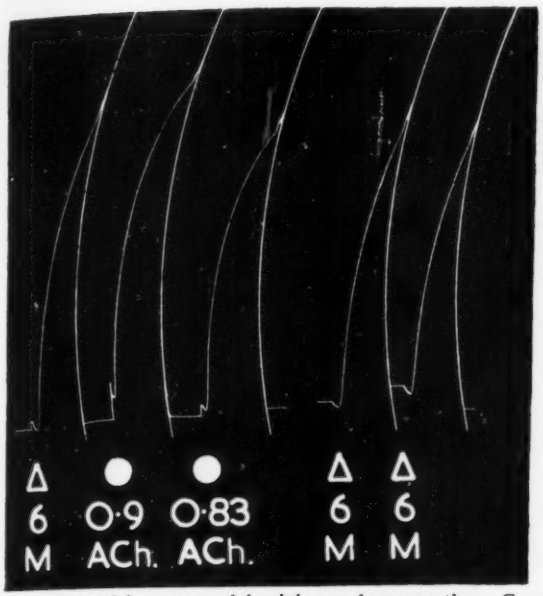

FIG. 1.—Isolated frog rectus abdominis muscle preparation. Comparison of the contractures elicited by a constant dose of murexine (M) with those following two different doses of acetylcholine (ACh). Bath volume, 2 ml. Contact time, 90 sec. The numerals indicate the dose of the drugs used in μg.

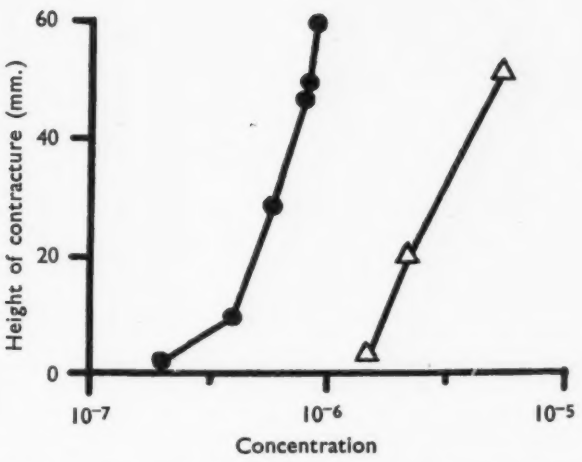

FIG. 2.—Dose/response curves obtained from an experiment with an isolated frog rectus muscle. Ordinate, height of contractures in mm. Abscissa, concentration of the drug used. ●=acetylcholine. Δ=murexine.

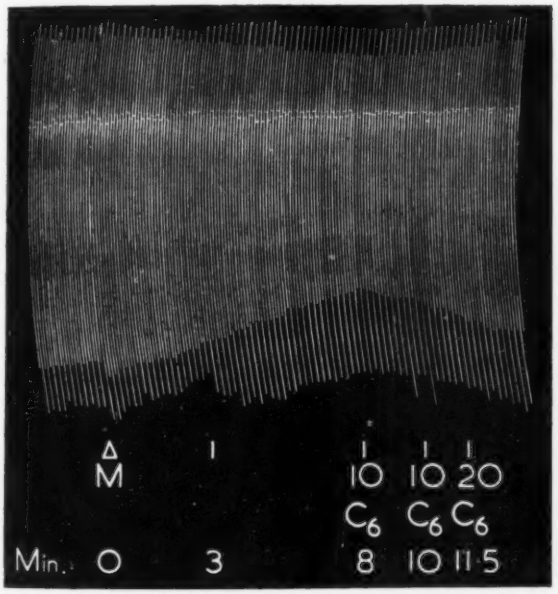

FIG. 3.—Isolated rat phrenic nerve-diaphragm preparation. Alternate stimulation of nerve and muscle. The twitches arising from direct simulation of the muscle are greater in amplitude than the indirect responses. 0.75 mg. murexine (M) was added at time 0. The response to nerve stimulation first suffered slight augmentation for five shocks and then was diminished in amplitude while the response to direct muscle stimulation was unaltered. At 8, 10, 11.5 min., 10, 10, and 20 mg. of hexamethonium (C_6) were added which antagonized the block. Bath volume, 50 ml. Temperature, 35°.

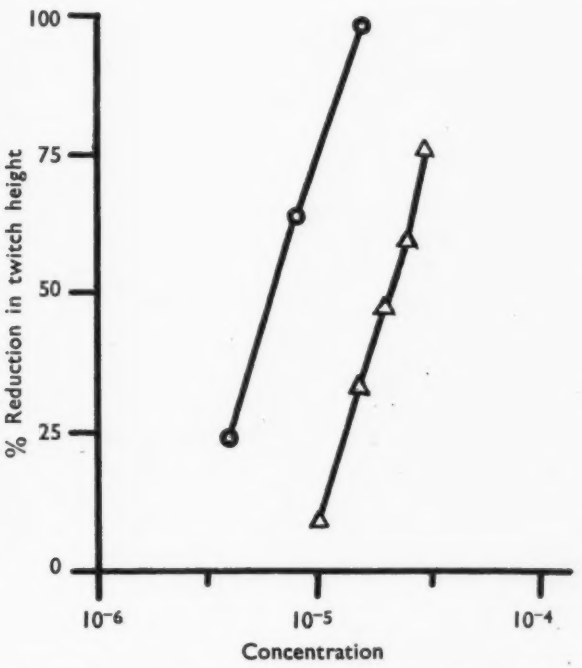

FIG. 4.—Dose/response curves obtained from an experiment with an isolated rat diaphragm. Ordinate, % reduction in twitch height. Abscissa, concentration of the drug used. O=suxamethonium. Δ=murexine.

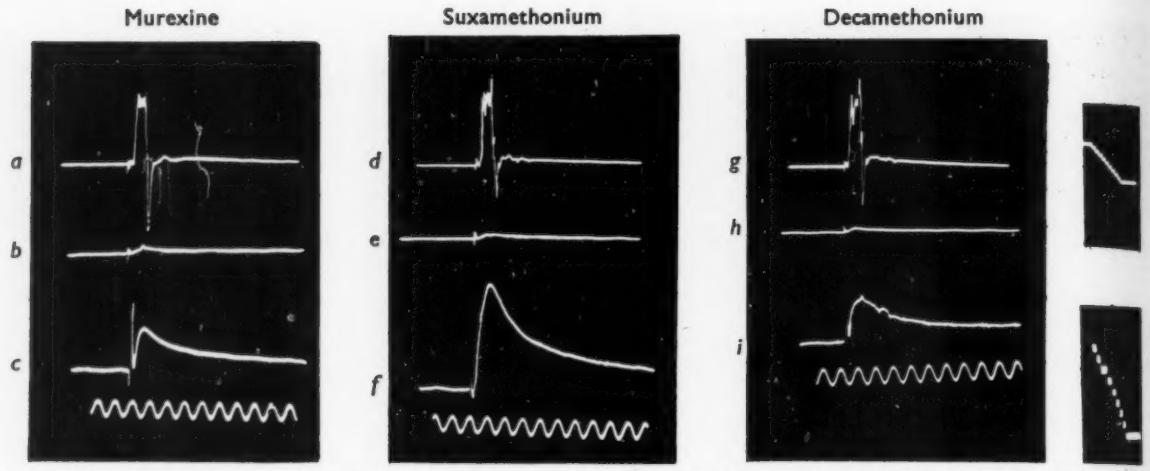

FIG. 5.—Electrical recordings made from the isolated frog *Extensor Longus Digiti IV Muscle* at the point of entry of its motor nerve. Comparison of the muscle action potential before (*a*, *d*, and *g*) with the end-plate potential after producing complete neuromuscular block (*b*, *c*, *e*, *f*, *h*, and *i*). Between (*a*) and (*b*) murexine 2×10^{-5}, (*d*) and (*e*) suxamethonium 1×10^{-6} and (*g*) and (*h*) decamethonium 2×10^{-5} were added to the fluid bathing the muscle to block neuromuscular transmission. The respective end-plate potentials were recorded with the same amplification used for the muscle action potentials (*b*, *e*, and *h*). Records of the end-plate potentials at greater amplification are given in (*c*), (*f*), and (*i*). The records in (*b*) and (*i*) are modified by a muscle action potential from at least one unblocked neuromuscular junction. At the amplification used for the top and middle rows of records, a calibration (upper right) in 1 mV. steps is given. The calibration for records (*c*) and (*i*) in 100 μV. steps is given in the lower right part of the figure. The amplification used for record (*f*) was three times more than that used in (*c*). Time signal, 100 c./sec.

three times less potent in producing neuromuscular block than was suxamethonium. The dose/response curves (Fig. 4) for suxamethonium (open circles) and for murexine (open triangles) were parallel, but that for murexine was displaced by about 0.5 log. units to the right of the curve for suxamethonium.

Electrical Studies with Isolated Extensor Longus Digiti IV Muscle *of the Frog*

The foot muscle was mounted vertically in a bath of frog Ringer containing 1×10^{-6} neostigmine with its distal extremity uppermost. For recording purposes one non-polarizable electrode was placed upon its distal end and another in the fluid below the muscle.

Neuromuscular Block.—Murexine in a concentration of 10 to 20 μg./ml. produced a complete neuromuscular block which was recognized by an absence of a twitch and of the muscle action potential when the motor nerve was stimulated and by the presence of an end-plate potential localized to the region around the point of entry of the nerve. In the foot muscle, most of the end-plates are located in this region. The features of the block with murexine are illustrated in Fig. 5*a*, *b*, and *c* and are compared with the block seen with appropriate concentrations of suxamethonium and decamethonium in the same muscle. All the records in Fig. 5 were made when the bath fluid was lowered to the point of entry of the nerve into the muscle. The upper trace in each section is a record of the normal muscle action potential (Fig. 5*a*, *d*, and *g*). The middle traces are records of the end-plate potential at the same point on the surface of the muscle when completely blocked with 2×10^{-5} murexine (Fig. 5*b*), 1×10^{-6} suxamethonium (Fig. 5*e*) and 2×10^{-5} decamethonium (Fig. 5*h*). The lowermost traces are amplified records of the end-plate potentials seen in the middle traces. When acetylcholine was added to the bath fluid in a concentration which caused complete neuromuscular block within 2 min., similar electrical results were observed.

Depolarization.—The moving fluid electrode technique was used to record the depolarizing action of compounds upon the muscle. By allowing the fluid to run out of the bath at a constant rate and recording on moving film, it was possible to obtain a record of the potential at each point along the surface of the muscle with reference to the electrode on its distal end. Such records are shown in Fig. 6 in which the horizontal axis corresponds to distance from the distal end of the muscle. The lowermost records in each group were made when normal frog Ringer bathing the muscle was allowed to run out of the bath. The small deflexions indicate little injury to the muscle cells. When a depolarizing drug was added to the bath fluid, depolarization developed

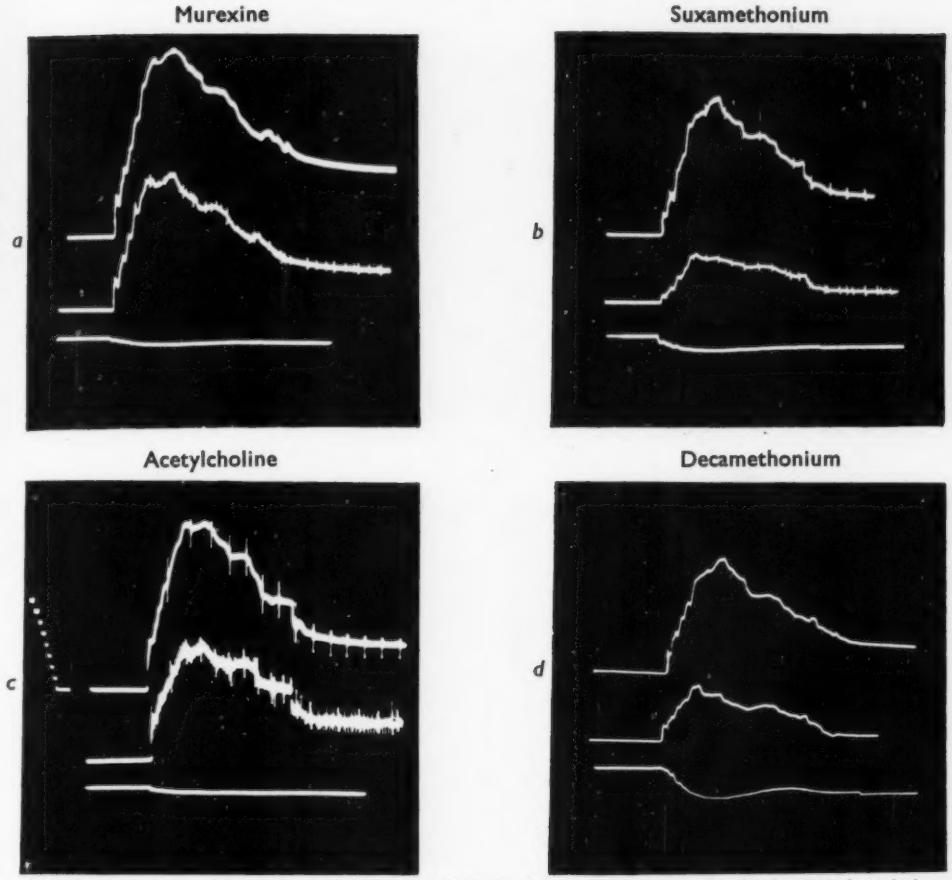

FIG. 6.—Records of depolarization of the *Extensor Longus Digiti IV Muscle* made with the moving fluid electrode technique as the solution ran out of the bath. The ordinate is voltage and a calibration signal in 1 mV. steps is provided on the left of record (*c*). Depolarization is shown by a vertical deflexion of the trace. The lowest trace in each group was made when frog Ringer solution ran out of the bath and shows that there was little injury to the muscle during dissection. The middle traces give the voltages of depolarization at 0.5 min. and the upper traces the maximum response after 2 min. exposure to depolarizing drugs. The peak of each depolarization record corresponded to the end-plate focus in the muscle. Asynchronous muscle action potentials fired off by the depolarization appear as brief vertical deflexions of the record. These potentials are most profuse in the early stages of depolarization represented by the middle traces in each group. The depolarizing agents used were in (*a*) murexine 2×10^{-5}, in (*b*) suxamethonium 1×10^{-6}, in (*c*) acetylcholine 1×10^{-6}, and in (*d*) decamethonium 2×10^{-5}. All the drugs produced substantial depolarization and asynchronous muscle action potentials although those with decamethonium were not photographed.

and was recorded as an upward deflexion of the trace. The middle record in each group shows the degree of depolarization which had developed in 0.5 min. and the upper records are of the maximum depolarization which was attained in 2 min. In all the experiments in which the muscles were in good condition, depolarizing drugs also gave rise to asynchronous muscle action potentials which were observed on the oscilloscope and were recorded upon the " depolarization sweeps " as in Fig. 6. The potentials were particularly in evidence during the early phase of the depolarizing action (Fig. 6 middle traces).

Murexine (2×10^{-5}) produced a marked depolarization of the foot muscle closely similar to that produced by appropriate concentrations of suxamethonium, acetylcholine and decamethonium (Fig. 6). Repeated washing restored the polarization of the muscle membrane after exposure to each of the four substances.

The voltage of depolarization produced by a drug is proportional to its concentration, and the dose/response curves for acetylcholine, suxamethonium, murexine, and decamethonium using the same muscle are given in Fig. 7. The curves for acetylcholine (solid circles) and for suxamethonium (open circles) are similar, being parallel to one another and lying in the same region of the graph. Those for murexine (hollow triangles) and decamethonium (solid triangles) fall in close proximity to one another and are approximately parallel to the curves for acetylcholine and suxamethonium but are displaced by about 1 log. unit to the right.

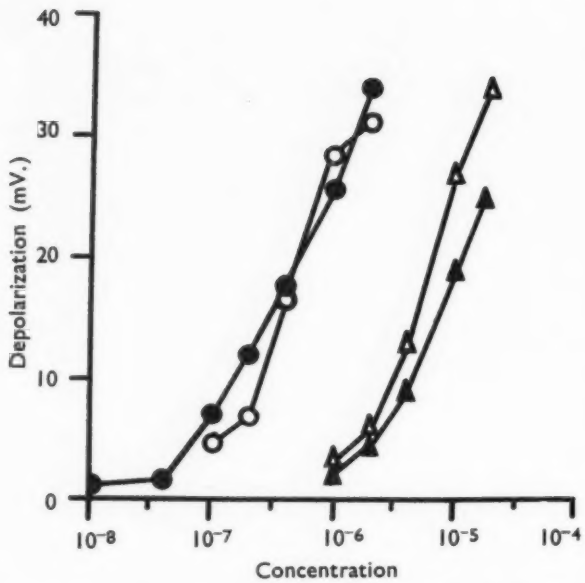

FIG. 7.—Dose/response curves relating voltage of depolarization of the *Extensor Longus Digiti IV Muscle* of the frog with the concentration of the drug used. ●=acetylcholine, ○=suxamethonium, △=murexine, and ▲=decamethonium.

DISCUSSION

The results of the experiments on isolated tissues are in accord with the findings of Erspamer and Glässer (1957) that murexine can produce a block of vertebrate neuromuscular transmission. The contracture response in the rectus and the relief of the block in the rat diaphragm by hexamethonium suggested that murexine was a "depolarizing type" of blocking agent. As the dose/response curves obtained from the frog rectus and from the rat diaphragm for suxamethonium and murexine were parallel but separated by about 0.5 log. units, it was inferred that murexine blocked by the same type of process as did suxamethonium but was less potent.

With the electrical experiments, it was possible to show that, at the moment of complete neuromuscular block following murexine, the absence of any muscle twitch in response to nerve stimulation was accompanied by an abolition of the muscle action potential which was replaced by an end-plate potential localized to the end-plate focus in the foot muscle. When the same muscle was exposed to concentrations of suxamethonium, of decamethonium, or of acetylcholine which produced complete neuromuscular block, the events observed with a murexine block were mimicked.

Using the moving fluid technique, it was found by direct measurement that murexine depolarized the foot muscle in a manner closely similar to that seen with acetylcholine, suxamethonium, and decamethonium. Murexine depolarization also gave rise to asynchronous muscle action potentials as did the other three depolarizing drugs. The slope of the curve relating the voltage of depolarization with the concentration in the case of murexine was parallel to those found with the other three agents. The depolarizing potency of murexine on the foot muscle of the frog was about ten times less than that of acetylcholine and of suxamethonium but was equal to that of decamethonium.

It was concluded that murexine could be classified as a "depolarizing type" of neuromuscular blocking agent but that it was three to ten times less potent than suxamethonium.

The author wishes to thank Professor V. Erspamer for the gift of the murexine chloride hydrochloride used in these experiments, Dr. J. G. Nicholls for help and advice, Mr. P. M. G. Bell for designing and constructing special electronic equipment, and Mr. K. Didcock for his unstinted technical assistance.

REFERENCES

Bell, P. M. G. (1957). *J. Physiol.*, **137**, 1P.
Bülbring, E. (1946). *Brit. J. Pharmacol.*, **1**, 38.
Erspamer, V. (1948). *Experientia*, **4**, 226.
—— and Glässer, A. (1957). *Brit. J. Pharmacol.*, **12**, 176.
Nicholls, J. G., and Quilliam, J. P. (1956). Ibid., **11**, 151.

Brit. J. Pharmacol. (1957), **12**, 393.

SUSCEPTIBILITY OF CERTAIN ENZYMES OF THE CENTRAL NERVOUS SYSTEM TO TETRODOTOXIN

BY

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The neurotoxic poison from the roe of Fugu, a globe fish living in the waters of Japan, was investigated with reference to its effect on the main oxidation-reduction systems, acetyl-cholinesterase and choline-acetylase in the brain of the guinea-pig.

It was found that the poison inhibited the choline-acetylase activity, even in a concentration of 4.4×10^{-7}. Acetyl-cholinesterase and cytochrome-c-oxidase activities were also inhibited in concentration of 1.3×10^{-5} and 0.8×10^{-5} respectively.

The Fugu, a poisonous kind of globe fish living in the home waters of Japan, causes several hundreds of cases of lethal poisoning every year, because the inhabitants eat it with relish, in spite of the high toxicity of some of its organs, particularly the ovaries. Hypaesthesia and paraesthesia are the first symptoms of the poisoning; motor paralysis follows; the patients die from paralysis of the respiratory centre and of the respiratory muscles, especially the diaphragm.

As it is clear from the symptoms that the poison, tetrodotoxin, affects the nervous system, its pharmacological actions on this system have been extensively studied (Kuriaki, Hiyoshi and Mochizuki, 1956). Yamagata (1940) claimed that tissue respiration and dehydrogenase activity in the brain of the cock and the mouse were inhibited by low concentrations and stimulated by higher concentrations of tetrodotoxin. Torikai (1944) observed that 0.3% of tetrodotoxin stimulated the activity of the succinic acid dehydrogenase of the rabbit heart and that xanthine dehydrogenase was markedly stimulated after a latent period. Some results of our studies on the biochemical aspects of its action are reported here.

METHODS

The poison of Fugu has now been successfully purified and has been obtained in crystalline form. The minimum lethal dose in mice is 0.01 to 0.001 mg./kg. body weight. The empirical formula is $C_4H_7O_3N$ (Yokowo, 1947; Tsuda and Kawamura, 1952). However, as the crystalline poison was not available to us, we used tetrodotoxin, a concentrate prepared by Sankyo Co. The method of preparation was precipitation of the concentrated aqueous extract of Fugu roe by lead acetate in an alkaline medium. Lead acetate in methanolic ammonia was then added to the concentrated solution of the precipitate in acidified water (10% H_2SO_3). The preparation was centrifuged and methanolic ammonia was added to the supernatant. The precipitate was dissolved in water and the solution treated with hydrogen sulphide and filtered. The filtrate was evaporated to dryness *in vacuo*. 1 g. of this concentrate corresponded in its toxic potency for the mouse to about 3 mg. of the pure crystalline poison.

In order to compare the effects of the poison on various enzymes and metabolites in the same tissue, all experiments were performed on homogenates of adult guinea-pig brain, except those on the yellow enzyme, which was derived from pig heart.

For determination of dehydrogenase activity, triphenyltetrazolium chloride (TTC) was employed in place of methylene blue, because, in contrast to the latter reagent, TTC when reduced gives a distinct red colour which is stable in the presence of oxygen. Sodium lactate, sodium succinate, sodium citrate, sodium pyruvate, and sodium β-glycerophosphate were used as substrates. One part of the brain of the guinea-pig was homogenized by means of a Potter homogenizer with five parts of phosphate buffer solution (0.2 M, *p*H 7.4). Each test-tube contained 1.0 ml. of the solution of tetrodotoxin, or 1.0 ml. of distilled water in the control group, 1.0 ml. of the solution of a substrate, 1.0 ml. brain homogenate, 1.0 ml. of 0.5% TTC reagent, and 2.0 ml. phosphate buffer solution. The test-tubes were filled with coal gas, tightly stoppered, and incubated at 38° for 2 hr. The reaction was stopped by addition of 1/10 volume of 20% trichloracetic acid. The triphenylformazan dye produced was extracted with 7.0 ml. of ethylacetate and the optical density was measured spectrophotometrically at a wavelength of 480 mμ.

For experiments on yellow enzyme, flavin adenine dinucleotide (FAD) was prepared from the heart

TABLE I

EFFECT OF TETRODOTOXIN ON ACTIVITIES OF DEHYDROGENASES FROM GUINEA-PIG BRAIN *IN VITRO*

Activity is expressed as amount of triphenylformazan dye produced in μg./g. wet weight of the brain.

No. of Expts.	Substrates	Conc. of Tetrodotoxin					
		0	$1{\cdot}7\times10^{-3}$	$1{\cdot}7\times10^{-4}$	$1{\cdot}7\times10^{-5}$	$1{\cdot}7\times10^{-6}$	$1{\cdot}7\times10^{-7}$
7	Succinate ..	$10{\cdot}57\pm2{\cdot}99$	$12{\cdot}15\pm2{\cdot}7$ ($0{\cdot}8>P>0{\cdot}7$)	$11{\cdot}6\pm3{\cdot}4$ ($0{\cdot}9>P>0{\cdot}8$)	$10{\cdot}5\pm2{\cdot}45$ ($0{\cdot}9>P>0{\cdot}8$)	$7{\cdot}8\pm2{\cdot}3$ ($0{\cdot}4>P>0{\cdot}3$)	$7{\cdot}35\pm1{\cdot}65$ ($0{\cdot}4>P>0{\cdot}3$)
7	Lactate ..	$9{\cdot}25\pm2{\cdot}55$	$15{\cdot}0\pm5{\cdot}8$ ($0{\cdot}5>P>0{\cdot}4$)	$8{\cdot}0\pm2{\cdot}0$ ($0{\cdot}8>P>0{\cdot}7$)	$8{\cdot}25\pm2{\cdot}2$ ($0{\cdot}8>P>0{\cdot}7$)	$8{\cdot}5\pm4{\cdot}15$ ($0{\cdot}9>P>0{\cdot}8$)	$8{\cdot}25\pm2{\cdot}3$ ($0{\cdot}8>P>0{\cdot}7$)
7	Pyruvate	$8{\cdot}27\pm2{\cdot}44$	$14{\cdot}5\pm6{\cdot}0$ ($0{\cdot}4>P>0{\cdot}3$)	$6{\cdot}0\pm2{\cdot}45$ ($0{\cdot}6>P>0{\cdot}5$)	$7{\cdot}8\pm1{\cdot}65$ ($0{\cdot}9>P>0{\cdot}8$)	$8{\cdot}35\pm4{\cdot}45$ ($0{\cdot}9>P>0{\cdot}8$)	$7{\cdot}15\pm2{\cdot}85$ ($0{\cdot}8>P>0{\cdot}7$)
7	Glycero-phosphate	$9{\cdot}26\pm2{\cdot}92$	$11{\cdot}0\pm2{\cdot}25$ ($0{\cdot}7>P>0{\cdot}6$)	$10{\cdot}3\pm3{\cdot}35$ ($0{\cdot}8>P>0{\cdot}7$)	$6{\cdot}15\pm2{\cdot}1$ ($0{\cdot}5>P>0{\cdot}4$)	$8{\cdot}5\pm3{\cdot}1$ ($0{\cdot}9>P>0{\cdot}8$)	$8{\cdot}5\pm3{\cdot}2$ ($0{\cdot}9>P>0{\cdot}8$)
7	Citrate	$7{\cdot}43\pm2{\cdot}59$	$8{\cdot}8\pm2{\cdot}3$ ($0{\cdot}8>P>0{\cdot}7$)	$8{\cdot}45\pm3{\cdot}2$ ($0{\cdot}8>P>0{\cdot}7$)	$9{\cdot}9\pm4{\cdot}45$ ($0{\cdot}7>P>0{\cdot}6$)	$6{\cdot}25\pm3{\cdot}8$ ($0{\cdot}9>P>0{\cdot}8$)	$6{\cdot}3\pm2{\cdot}05$ ($0{\cdot}9>P>0{\cdot}8$)

muscle of the pig according to the description of Lockhart (1930) and von Euler and Hellström (1938). Preparation of reduced diphosphopyridine nucleotide (DPN) was performed according to Green, Dewan, and Leloir (1937). The experimental procedure of Greig (1946) was followed, except that TTC was used instead of methylene blue as the hydrogen acceptor.

Cytochrome oxidase activity was determined by a simple method which consisted of immersing pieces of sliced and frozen guinea-pig brain in a solution of Nadi reagent for 15 min. and comparing their colour density macroscopically with that of pieces put in the Nadi reagent containing tetrodotoxin (Becker, 1949).

The method of Feldberg (1950) was followed for determination of choline-acetylase activity, and effect *in vitro* of the poison on the enzyme was studied. The procedure of von Muralt (1942) with the frog rectus muscle was used for the evaluation of acetylcholine production.

Acetyl-cholinesterase activity of the brain homogenate and effect of the poison on it were determined manometrically according to the method of Ammon (1934). 0.5 ml. of 0.0025 M acetylcholine chloride was placed in the side arm. Homogenate of 0.3 g. brain of the guinea-pig in 3.0 ml. of 0.025 M Ringer-bicarbonate solution and 0.5 ml. of tetrodotoxin solution of various concentrations were placed in the main compartment of the flask.

Determination of high energy phosphates was carried out as follows: the brain of the normal guinea-pig and that of the guinea-pig administered sub-lethal dose of tetrodotoxin (0.45 mg.) were homogenized at a temperature below 0° by cooling from outside the homogenizer with dry ice and methanol. The high energy phosphates were fractionated by paper chromatography, as described by Fleckenstein (1952, 1953) and Gerlach, Weber, and Döring (1955). Extracts of the paper were treated as described by Berenblum and Chain (1938), and the optic densities were determined spectrophotometrically.

RESULTS

As Table I indicates, the Fugu poison in concentrations of 10^{-7} to 10^{-3} did not have any significant effect on activities of the dehydrogenases when incubated with various substrates *in vitro*.

Experiments were also performed on the dehydrogenases *in vivo*, injecting a guinea-pig first with 0.45 mg. (sub-lethal dose) of the poison subcutaneously; then 25 min. later with 1.0 ml. of 1% TTC solution in saline in the carotid artery. The brain was excised 10 min. later and its colour density was compared with that of an untreated brain of a control animal. There was no apparent difference in the colour density of the two brains.

The yellow enzyme was not affected by the poison in concentrations of 10^{-7} and 10^{-4}, as shown in Table II.

TABLE II

EFFECT OF TETRODOTOXIN ON THE YELLOW ENZYME ACTIVITY *IN VITRO*

Activity is expressed as amount of triphenylformazan dye produced in μg./g. wet weight of the brain.

No. of Expts.	Conc. of Tetrodotoxin		*P* Value
8	0 (control)	$5{\cdot}13\pm0{\cdot}36$	
8	$1{\cdot}1\times10^{-7}$	$5{\cdot}21\pm0{\cdot}32$	$0{\cdot}9>P>0{\cdot}8$
8	$1{\cdot}1\times10^{-6}$	$5{\cdot}21\pm0{\cdot}32$	$0{\cdot}9>P>0{\cdot}8$
8	$1{\cdot}1\times10^{-5}$	$5{\cdot}25\pm0{\cdot}32$	$0{\cdot}9>P>0{\cdot}8$
8	$1{\cdot}1\times10^{-4}$	$5{\cdot}31\pm0{\cdot}33$	$0{\cdot}9>P>0{\cdot}8$

On cytochrome oxidase, the poison was ineffective in concentrations of 10^{-8} to 10^{-6}, but effective in concentrations of 10^{-5}, depressing the activity. In concentrations of 10^{-4} to 10^{-3}, inhibition was marked, as seen in Table III.

TABLE III

EFFECT OF TETRODOTOXIN *IN VITRO* ON CYTOCHROME OXIDASE ACTIVITY OF GUINEA-PIG BRAIN

Activity was estimated as degree of coloration of Nadi reagent and intensity is proportional to the number of symbols +.

No. of Expts.	Conc. of Tetrodotoxin	
8	0 (control)	+++
8	$0{\cdot}8\times10^{-8}$	+++
8	$0{\cdot}8\times10^{-7}$	+++
8	$0{\cdot}8\times10^{-6}$	++
8	$0{\cdot}8\times10^{-5}$	–
8	$0{\cdot}8\times10^{-4}$	–
8	$0{\cdot}8\times10^{-3}$	–

Choline-acetylase activity was inhibited by the poison in concentrations of 10^{-6} and 10^{-7}, 0.128 and 0.133 mg. of acetylcholine being produced respectively/g. acetone powder of the guinea-pig brain, in contrast to 0.221 mg./g. acetone powder in the control. In concentrations of 10^{-9} to 10^{-8}, the poison had no significant effect on the enzyme (Table IV).

TABLE IV

EFFECT OF TETRODOTOXIN *IN VITRO* ON CHOLINE-ACETYLASE ACTIVITY OF GUINEA-PIG BRAIN

Activity is expressed as amount of acetylcholine produced in mg./g.

No. of Expts.	Conc. of Tetrodotoxin		*P* Value
10	0 (control)	0·221	
10	$4{\cdot}4 \times 10^{-6}$	0·128	$0{\cdot}02 > P > 0{\cdot}01$
10	$4{\cdot}4 \times 10^{-7}$	0·133	$0{\cdot}05 > P > 0{\cdot}02$
10	$4{\cdot}4 \times 10^{-8}$	0·194	$0{\cdot}8 > P > 0{\cdot}7$
10	$4{\cdot}4 \times 10^{-9}$	0·209	$0{\cdot}9 > P > 0{\cdot}8$

Acetyl-cholinesterase was noticeably inhibited by tetrodotoxin in high concentrations of 1.3×10^{-5} and 1.3×10^{-4}. The Q_{CO_2} was 14.43 and 9.46 ml./mg./hr. respectively, whereas it was 26.67 ml./mg./hr. in the control. In 1.3×10^{-6} of tetrodotoxin, the Q_{CO_2} was 19.72, but the difference from that of the control was not statistically significant. 1.3×10^{-7} and 1.3×10^{-8} were no more effective, Q_{CO_2} being 28.31 and 32.72 respectively (see Table V).

Contents of high energy phosphates in normal guinea-pig brain were compared with those in the brain of the guinea-pig given a sub-lethal dose of tetrodotoxin (0.45 mg.). It can be seen from Table VI that the content of creatine phosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and inorganic phosphate in guinea-pig brain was not significantly altered by a sub-lethal dose of tetrodotoxin.

TABLE V

EFFECT *IN VITRO* OF TETRODOTOXIN ON ACETYL-CHOLINESTERASE ACTIVITY OF GUINEA-PIG BRAIN

Activity is expressed as amount of CO_2 evolved from $NaHCO_3$ in consequence of acetic acid formation by the enzyme.

No. of Expts.	Conc. of Tetrodotoxin	Q_{CO_2} µl./mg./hr.	*P* Value
10	0 (control)	26·67	
10	$1{\cdot}3 \times 10^{-4}$	9·46	$0{\cdot}02 > P > 0{\cdot}01$
10	$1{\cdot}3 \times 10^{-5}$	14·43	$0{\cdot}05 > P > 0{\cdot}02$
10	$1{\cdot}3 \times 10^{-6}$	19·72	$0{\cdot}2 > P > 0{\cdot}1$
10	$1{\cdot}3 \times 10^{-7}$	28·31	$0{\cdot}8 > P > 0{\cdot}7$
10	$1{\cdot}3 \times 10^{-8}$	32·71	$0{\cdot}7 > P > 0{\cdot}6$

TABLE VI

EFFECT OF TETRODOTOXIN *IN VIVO* ON THE AMOUNT OF HIGH ENERGY PHOSPHATES IN THE GUINEA-PIG BRAIN

No. of Expts.	Procedure (See Text)	Brain of Guinea-pig Injected 0·45 mg. of Tetrodotoxin (µg./g.)	Control (µg./g.)	*P* Value
6	CP	35·48	37·67	$0{\cdot}8 > P > 0{\cdot}7$
6	ATP and ADP	65·49	56·71	$0{\cdot}7 > P > 0{\cdot}6$
6	Inorganic phosphate	263·99	241·61	$0{\cdot}2 > P > 0{\cdot}1$

DISCUSSION

It follows from these results that, of those enzymes studied, choline-acetylase was most sensitive to the poison. This fact may be noteworthy, even though these *in vitro* experiments may not lead to any direct conclusions on the mechanism of action of the poison. It resembles Botulinus toxin, which is assumed by Burgen, Dickens and Zatman (1949), Ambache (1949, 1951), Brooks (1954) and others to attack the end of the cholinergic nerve fibre, reducing the production of acetylcholine there. The concentration of the poison, 4.4×10^{-7}, which depresses the enzyme activity *in vitro*, would easily be attained *in vivo* in cases of poisoning. Fukuda and Tani (1941) have observed in the cases of poisoning that a dose of 10 g. of Fugu ovaries was a lethal dose for man. As 1 g. of ovary can kill 20 kg. of mice and the minimum lethal dose of the crystalline poison is 0.01 µg./g., the amount of toxin present in ovary is of the order of 1 part in 5,000. Thus, the minimum lethal dose for man can be computed as approximately 0.03 µg./g. Since 1 g. of the poison concentrate which we used would correspond to about 3 mg. of the crystalline poison in its toxicity for mice, the threshold concentration of 4.4×10^{-7} would be attained in the body.

Where used in higher concentrations than in the choline-acetylase experiments, the poison also depresses the activity of cytochrome oxidase and that of acetyl-cholinesterase, but it does not affect the dehydrogenases or the yellow enzyme. Therefore, it is unlike anaesthetics, the action of which is attributed by Quastel (1951) to their inhibiting effect on flavoprotein activity. It is also unlike the bee- and snake-poisons which specifically inhibit dehydrogenase activity in markedly low concentrations (Fleckenstein and Janke, 1953). These results do not agree with those of Yamagata (1940), who claimed effects on tissue respiration and dehydrogenase activity. In any case, it is unlikely that low concentrations would inhibit and higher concentrations would stimulate. We are also unable to confirm Torikai (1944), who reported stimulation of dehydrogenases by tetrodotoxin.

Nagazawa and Nagai (personal communication) have compared the amount of acid-soluble phosphates in the brain and liver of normal rats, as well as in rats killed with tetrodotoxin ; they found no difference between normal and poisoned organs. Our results obtained by the new method agree with theirs, in that the content of high energy phosphates in the brain was not affected by the poison.

References

Ambache, N. (1949). *J. Physiol.*, **108**, 127.
—— (1951). Ibid., **113**, 1.
Ammon, R. (1934). *Pflüg. Arch. ges. Physiol.*, **233**, 486.
Becker, V. (1949). *Arch. exp. Path. Pharmak.*, **207**, 109.
Berenblum, I., and Chain, E. (1938). *Biochem. J.*, **32**, 295.
Brooks, V. B. (1954). *J. Physiol.*, **123**, 501.
Burgen, A. S. V., Dickens, F., and Zatman, L. J. (1949). Ibid. **109**, 10.
Euler, H. von, and Hellström, H. (1938). *Z. phys. Chem.*, **252**, 31.
Feldberg, W. (1950). *Methods in Medical Research*, **3**, 95. Chicago: Year Book Publishers.
Fleckenstein, A. (1952). *Arch. exp. Path. Pharmak.*, **216**, 184.
—— (1953). Ibid., **219**, 531.
—— and Janke, J. (1953). *Pflüg. Arch. ges. Physiol.*, **258**, 177.
Fukuda, T., and Tani, Y. (1941). *Nippon Igaku*, **3258**, 2739.
Gerlach, E., and Weber, E. (1955). *Arch. exp. Path. Pharmak.*, **224**, 496.
—— —— and Döring, H. J. (1955). Ibid., **226**, 9.
Green, D. E., Dewan, J. G., and Leloir, L. F. (1937). *Biochem. J.*, **31**, 934.
Greig, M. E. (1946). *J. Pharmacol.*, **87**, 185.
Kuriaki, K., Hiyoshi, K., and Mochizuki, Y. (1956). *Jap. J. Pharmacol.*, **6**, 37.
Lockhart, E. (1930). *Biochem. J.*, **33**, 613.
Muralt, A. von (1942). *Pflüg. Arch. ges. Physiol.*, **245**, 604.
Quastel, J. H. (1951). *Mecanisme de la Narcose, Colloques internationaux du Centre National de la Recherche Scientifique, Paris*, p. 105.
Torikai, A. (1944). *Rinsho to Kenkyu*, **21**, 361.
Tsuda, K., and Kawamura, M. (1952). *J. pharm. Soc., Japan*, **72**, 187, 771.
Yamagata, T. (1940). *Igaku Kenkyu*, **14**, 2155.
Yokowo, A. (1947). *Riken Iho*, **24**, 136.

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A METHOD OF ESTIMATING HISTAMINE IN PLASMA

BY

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The method is intended for the estimation of minute quantities of histamine in blood plasma. A neutralized trichloracetic acid extract of plasma is adsorbed at *p*H 8 on a column prepared by mixing a quantity of the cationic exchange resin Amberlite XE-64 with powdered cellulose as a supporting medium. Histamine is adsorbed but *N*-acetylhistamine is not: the amines can therefore be estimated independently. Elution of the histamine is by displacement with HCl. The eluate is converted into the solution for bioassay on the superfused guinea-pig ileum.

In recovery experiments histamine was added in the range 25 to 100 ng. to solutions of known composition and to plasma (5 ml.) obtained from man and the cat. The mean recovery in this range for histamine added to plasma was 82.5% ±2 S.E. (17 estimations).

The histamine equivalent of human plasma obtained from the antecubital vein was found to be less than 1 ng./ml.

It was possible by this method to follow the concentration of histamine in the arterial plasma of the cat when histamine was infused intravenously at a rate of 330 ng./kg./min. There was no perceptible change in the arterial blood pressure during the infusion, but the plasma histamine rose from <0.3 ng./ml. to 3.4 ng./ml. (mean of 2 estimations).

When the quantity of histamine extractable from plasma is less than can be estimated by conventional methods of bioassay, the sensitivity of the test can be increased by applying the undiluted extract directly to the strip of guinea-pig ileum; this may be suspended in a minute bath (Mongar and Schild, 1950) or in air, as in the technique of superfusion (Gaddum, 1953). The method is applicable if the histamine has been sufficiently purified and if the test solution is similar in composition to the solution bathing the intestine between the doses.

If plasma is extracted by the method of Barsoum and Gaddum (1935) as modified by Code (1937a) and tested in this way, the activity of the extract is only partially abolished by the histamine antagonist mepyramine maleate (Mongar and Whelan, 1953). Moreover, the method does not distinguish between free histamine and histamine which may be released from pharmacologically inactive forms at the stage of refluxing the extract in strong acid (unpublished results).

Various chromatographic methods of purifying histamine have been proposed and are reviewed in the article by Code and McIntire (1956). Some employ paper chromatography, others weak cationic exchangers which are used to remove histamine from solutions containing organic solvents. These methods are designed mainly for the chemical determination of histamine, but some have been combined with bioassay.

In the method to be described the purification is conducted entirely in aqueous solution with the aid of a carboxylic ion exchange resin, at room temperature and at *p*H values not far from neutral. Advantage is taken of the fact that carboxylic resins have a high exchange capacity and can be buffered over a wide range of *p*H. They can therefore be used to separate weak organic bases in a mixture depending on whether or not the bases are ionized at the *p*H of the adsorption. Unionized bases and non-electrolytes remain in the aqueous phase. Since the resins have a high affinity for hydrogen ions, adsorbed cations can be readily eluted with acidic solutions (Kunin and Meyers, 1950; Samuelson, 1952; Bregman, 1953; Partridge, 1954).

The essential steps in the method are: (1) precipitation of the plasma proteins with trichloracetic acid followed by centrifugation, (2) preparation of an aliquot of the supernatant for adsorption on buffered columns of the resin (usually *p*H 8), (3) elution of histamine and other bases by displacement with HCl, and (4) conversion of the eluate proper into the solution for bioassay by

methods that have already been described (Adam, Hardwick, and Spencer, 1954).

The method at first depended on adsorbing the histamine on Amberlite IRC-50 at *p*H 5.9; later it was made more sensitive by performing the adsorption on Amberlite XE-64 at *p*H 8. Some of the results reported in this paper were obtained with IRC-50 at *p*H 5.9; details of this earlier procedure are therefore included in the sections that follow.

Materials and Methods

Resins.—Amberlite IRC-50 (Analytical Grade: B.D.H.) is supplied in the hydrogen form (R–COOH) and consists of moist, adherent beads. It was therefore dried at 50° until the beads ran freely. Amberlite XE-64 (Rohm and Haas Company, Philadelphia, Pa.) is chemically identical with IRC-50 but consists of a fine powder of unspecified particle size. A fraction of the powder containing the larger particles was obtained in the following way. A quantity of resin was suspended in water (30 g./l. in a beaker) and the suspension allowed to stand for 10 min. The particles remaining in suspension were poured off and the sediment was resuspended. This procedure was repeated until the supernatant was more or less clear (6 times). The coarse fraction obtained finally was dried at 50° for 24 hr. (There is now available "Amberlite chromatographic grade resin" CG-50 Type I and II (B.D.H.). Type I (100 to 200 mesh) corresponds fairly closely to the fraction of large particles obtained from XE-64.)

The exchange capacity of the resin for a metallic ion such as sodium is estimated to be 10 m.equiv./g. dry resin (Kunin and Meyers, 1950). This represents a maximum value obtained under conditions of high *p*H and ionic strength, and after prolonged contact with the ambient solution; in practice, it is nearer 9 m.equiv./g. dry resin. According to Bergström and Hansson (1951), 1 ml. IRC-50 (0.7 g. dry resin) adsorbs 190 mg. histamine at *p*H 7.2; the salt used is not mentioned.

Powdered cellulose (Whatman Standard Grade for Chromatography) was used as a supporting medium for columns containing XE-64. These are referred to as composite columns.

Solutions.—"AnalaR" materials were used. Stock buffers used in the method had the following composition: *p*H 7.92, 53 ml. 0.2 M NaH_2PO_4+947 ml. 0.2 M $NaHPO_4$ (calculated to be approximately 0.39 N in terms of Na^+). *p*H 5.9, 840 ml. 0.2 M NaH_2PO_4+160 ml. 0.2 M Na_2HPO_4 (calculated to be approximately 0.23 N in terms of Na^+). The solutions were standardized electrometrically and stored in the cold. Trichloracetic acid (TCA), 6% w/v. Hydrochloric acid, 0.1 N and 0.25 N; prepared from concentrated volumetric solution (B.D.H.). Sodium hydroxide, 0.1 N and 1 N. Neutral Red, 0.01% w/v aqueous solution.

Drugs.—Histamine acid phosphate (B.D.H.); the values quoted in this paper refer to the base on the assumption that this corresponds to 36.16% by weight of the salt.

N-acetylhistamine (4-(β-acetyl-amino-ethyl)-imidazole) m.p. 147° was prepared synthetically. (Tabor and Mosettig, 1949; Dr. C. R. Ricketts, personal communication).

Adrenaline (B.D.H.) and 5-hydroxytryptamine creatinine sulphate [5-HT] (May and Baker); the quantities are expressed as the base.

Heparin *B.P.* (Boots) 103 to 115 international units/mg.

Siliconing of Glass.—Glass pipettes for measuring exact volumes of plasma were freshly coated with silicone DC 1107 obtained from Midland Silicones.

Plastic and Other Materials.—Vessels and centrifuge tubes made of polythene were used throughout in handling the blood and plasma. Open tubes were covered with the non-wettable material "Parafilm" (Marathon Corporation, Menasha, Wis.) to keep out dust and to make mixing possible. Teat pipettes with upturned tips were drawn out from polythene tubing (o.d. 8 mm.) as described by Condon (1954) and were used to separate the plasma.

Preparation of Columns

The resin was first treated with NaOH so as to convert about the right proportion into the sodium salt for the *p*H required; the final adjustment of *p*H was obtained by equilibrating the column with buffer solution. The column was made in a glass tube (6 mm. i.d. × 170 mm.) surmounted by a bulb of 30 ml. capacity and closed by a tap. A small plug of glass wool was carefully packed into the bottom of the tube.

Composite Columns (*pH 8*).—50 mg. of the prepared XE-64 was intimately mixed with 300 mg. powdered cellulose in a 25 ml. stoppered, conical flask. 4 ml. 0.1 N NaOH was added from a burette and the suspension allowed to stand overnight at room temperature (if for longer, it was stored at 5° to lessen the danger of bacterial growth).

Stock buffer *p*H 7.92 (0.39 N Na^+) was diluted to 0.1 N or 0.15 N depending on whether the volume of plasma extracted was 2.5 ml. or 5 ml. The tube was filled with 4 ml. of the diluted buffer. 10 ml. of this buffer was added to the resin in the conical flask and the suspension poured into the bulb. When the column had begun to form, the tap was opened; the flow was finally adjusted to 0.3 ml./min. The column was 40 mm. high and had a volume of just over 1 ml. The total volume from the top of the column to the point of outflow was 1.5 ml. The column did not run dry and solutions were allowed to pass through until the tube above the column was empty. Buffer of the appropriate strength was applied to the column until the *p*H of the effluent reached that of the buffer (to within 0.1 unit); 15 ml. usually sufficed.

IRC-50 Columns (*pH 5.9*).—10 ml. 0.1 N NaOH was added to 325 mg. IRC-50 and the mixture allowed to

stand at room temperature for 24 hr. The resin was transferred to the tube with the aid of a wide-mouthed pipette and allowed to settle in the buffer. The height of the column was 38 mm. The solution above the column was drained off and the flow rate adjusted to 0.3 ml./min. Fresh buffer was then applied to the column until the *p*H of the effluent reached that of the buffer (to within 0.1 unit); 15 ml. usually sufficed.

Preliminary Experiments on Adsorption and Elution

The results on which the method is based are best expressed by curves showing the degrees of adsorption of sodium, histamine, and acetylhistamine over a range of *p*H. Experiments were also carried out to test the effect of sodium ion concentration on the adsorption of histamine and to follow the elution of histamine with HCl.

Sodium.—Columns made with 325 mg. IRC-50 were brought to a definite *p*H by treatment with buffer solution (Fig. 1). They were then washed with water and eluted with enough 0.1 N HCl to displace all the adsorbed Na^+. The difference between the acid concentration and the chloride concentration of the eluate was taken to represent the amount of sodium retained on the column. Similar results were obtained with composite columns containing XE-64 (30 mg.) mixed with cellulose (300 mg.). The curves (Fig. 1) show approximately the quantity of sodium ion which the resin will take up when it is equilibrated with buffer of known strength and *p*H, and consequently the quantity of sodium ion which the resin will release into the eluate when the column is neutralized with an excess of HCl.

Histamine and Acetylhistamine.—The curves shown in Fig. 2 relate % adsorption of 100 μg. histamine and

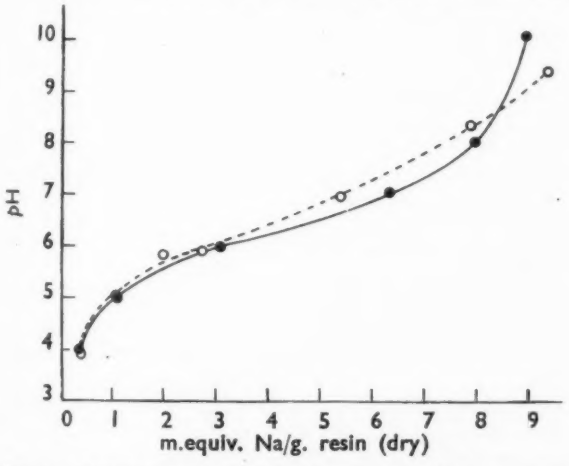

FIG. 1.—Adsorption/*p*H curves for sodium. Adsorption on composite columns containing Amberlite XE-64 (solid circles) and on columns made with Amberlite IRC-50 (open circles). The columns were treated with buffer solutions (0.2 N [Na^+]) until the *p*H of the effluent rose to that of the buffer. The sodium was eluted with HCl and determined as sodium chloride. Buffer solutions used: $CH_3COOH+CH_3COONA$ (*p*H 4 to 5), $NaH_2PO_4+Na_2HPO_4$ (*p*H 5.8 to 8), $H_3BO_3+NaCl+NaOH$ (*p*H 8 to 10), Na_2HPO_4+NaOH (*p*H 10 to 12).

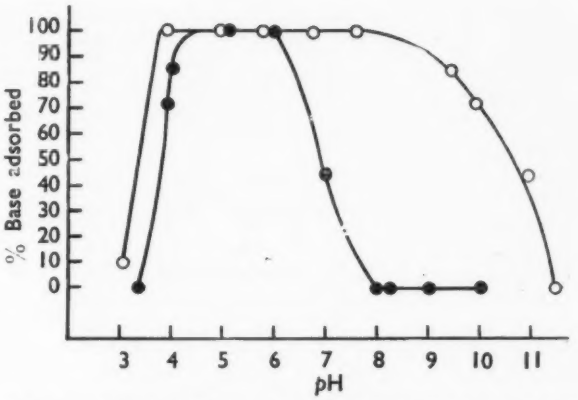

FIG. 2.—Adsorption/*p*H curves for 100 μg. histamine (open circles) and 100 μg. acetylhistamine (solid circles) on columns of Amberlite IRC-50. Buffer solutions used: HCl+potassium hydrogen phthalate (*p*H 3.2), $CH_3COOH+CHCOONa$ (*p*H 4 and 5), $NaH_2PO_4+Na_2HPO_4$ (*p*H 5.8 to 8), $H_3BO_3+NaOH+NaCl$ (*p*H 8 to 10), $NaOH+Na_2HPO_4$ (*p*H 10 to 12). All the solutions containing sodium were 0.2 N in terms Na^+.

of 100 μg. acetylhistamine on buffered columns of IRC-50 (1 g. dry resin: 6 mm. × 60 to 120 mm., depending on *p*H). The results were obtained by measuring the unadsorbed amines colorimetrically (Roberts and Adam, 1950). At *p*H 3.2 only a small fraction of the column is ionized and adsorption of the amines is slight. Histamine is completely adsorbed in the *p*H range 4 to 8; thereafter the adsorption declines as the ionization of the amine is suppressed. At the *p*H corresponding roughly to its $pK\alpha$ (9.7, Levy, 1935) about 80% is adsorbed. Acetylhistamine is completely adsorbed in the *p*H range 4.3 to 6 and not at all at *p*H 8. At this *p*H the two bases are completely separable. This result was confirmed with smaller quantities of the amines which were estimated biologically. The results are presented in Table I. Acetylhistamine was estimated as histamine after hydrolysis with HCl (Roberts and Adam, 1950). The residue obtained after removal of the HCl by evaporation was dissolved in water (10 ml.), buffered (5 ml. stock buffer *p*H 5.9) and applied to a column of IRC-50 (*p*H 5.9); this made it possible to separate hist-

TABLE I

ESTIMATES OF HISTAMINE AND ACETYLHISTAMINE AFTER SEPARATION FROM MIXTURES

Column	Buffer		Histamine			Acetylhistamine		
	*p*H	Normality of Na^+	Added	Eluted	%	Added	Unadsorbed	%
			μg.	μg.		μg.	μg.	
IRC-50 (500 mg.)	7	0·23	5	4·5	90	10	7·6	76
	8	0·23	5	5·2	108	10	8·6	86
	9	0·23	5	4·0	80	10	9·0	90
			ng.	ng.		ng.	ng.	
XE-64 (30 mg.)	8	0·12	50	40	80	200	144	72
+ Cellulose (300 mg.)	8	0·12	50	50	100	75	56	75

amine from phosphate ions (for details of method see below). (The variable recoveries for acetylhistamine were partly explained by losses incurred during these procedures. Control experiments showed that a loss of 20 to 50 ng. was likely to occur when 100 ng. histamine was subjected to the hydrolysis only.)

Effect of Sodium Ion Concentration.—10 ml. buffer containing 50 ng. histamine was applied to composite columns made with only 30 mg. XE-64. The columns were subsequently washed with water and eluted with HCl. The sodium ion concentration of the buffer varied from 0.04 to 0.4 N. In the range 0.04 N to 0.2 N, the mean recovery was 97% (range (5) 87 to 110); at 0.3 N the recovery was 60% and at 0.4 N only 40%. It was evident that the composite columns were critically sensitive to salt concentration and that when the volume applied to the column was 10 ml. the sodium ion concentration should not exceed 0.2 N.

Effect of Washing Buffered Columns with Water.—The *p*H of the effluent rose by 0.5 to 1.0 unit presumably owing to the diffusion of Na^+ into the aqueous phase: there was, however, no detectable loss of histamine at this stage.

Elution of Histamine with Acid.—100 ng. histamine was applied to a column of IRC-50 (325 mg.; *p*H 5.9). The column was washed with 5 ml. water and treated with 15 ml. 0.1 N HCl. The eluate was collected in three 5 ml. fractions. The sodium appeared mainly in the first and second fractions and the histamine in the second and third fractions after the *p*H had fallen to below 4. Since the third fraction (*p*H 1.1) contained mostly free acid, it was concluded that elution should be performed with a quantity of acid slightly exceeding the theoretical equivalent of base on the column.

Preparation of Plasma

Collection of Blood.—Human blood was obtained from the antecubital vein of healthy donors and allowed to run freely into a chilled polythene bottle containing 0.9% NaCl (10% by volume) and enough heparin to give a final concentration of about 10 units/ml. of the mixture. Cats were anaesthetized with sodium pentobarbitone (Veterinary Nembutal, Abbott) and given heparin (5 mg./kg. i.v.). Polythene tubing (i.d. 0.30 in. × o.d. 0.48 in.; Intramedic, Clay-Adams, Inc.) was introduced centrally into the femoral or carotid artery, and the blood flowed into graduated centrifuge tubes in ice containing 0.1 volumes of saline and enough heparin to give 5 units/ml. of the mixture.

Centrifugation.—The blood was centrifuged at 900 *g* for 10 min. (4°). The plasma was then separated and centrifuged at 2,200 *g* for 60 min. (4°).

5 ml. human plasma or 2.5 ml. cat plasma was placed in 10 ml. centrifuge tubes for extraction. In recovery experiments histamine or other drugs were added at this stage from a micrometer syringe. The drugs were dissolved in 0.9% NaCl and delivered in volumes of 0.1 to 0.2 ml. Mixing was obtained by inverting the tube several times.

Separation of Histamine

Precipitation of Plasma Proteins.—5 ml. TCA was added to 5 ml. centrifuged plasma. The mixture was well stirred and left at room temperature for 60 min. The precipitate was removed by centrifugation at 2,200 *g* for 30 min. (4°). The volume of supernatant (SN) obtained from different samples of plasma was 7.2 ± 0.2 (25).

Preparation of the Solution for Adsorption.—A 5 ml. aliquot of the SN was transferred to a 10 ml. stoppered, graduated tube. One drop of neutral red solution (0.01% w/v) was added as internal indicator. The free trichloracetic acid was neutralized with N NaOH (0.70 ml. $\pm$ 0.08 (25)), a very pale pink being taken as the end point. A final adjustment was usually made with 0.1 N HCl and 0.1 N NaOH. These solutions were run from microburettes (1 to 5 ml. capacity; Grade A) fitted with polythene adaptors which provided drops of 0.01 to 0.02 ml. The solution for adsorption was brought to *p*H 8 ± 0.05 by the addition of 1 ml. of buffer solution (*p*H 7.92) and the volume was made up to 10 ml. with water. The sodium ion concentration of this solution was calculated to be roughly 0.15 N on the assumption that each of the following contributed in m.equiv. sodium trichloracetate (NaTCA) 0.7, NaCl from the plasma 0.35 and 1 ml. buffer 0.39.

When the volume of plasma extracted was 2.5 ml., the volume of SN was 3.6 ml., of which 3 ml. was taken for the adsorption. The titratable TCA (0.42 m.equiv.) and the estimate of NaCl (0.21 m.equiv.) from the plasma were then proportionately less. The sodium ion concentration of the final solution (10 ml.) was calculated to be roughly 0.1 N.

A control solution containing NaCl and the reagents was prepared for adsorption in parallel with the plasma extract.

Adsorption and Elution.—The solution was applied to a composite column buffered at *p*H 8 which was then washed with 5 ml. water. The total effluent (15 ml.) was collected when it was desired to estimate acetylhistamine. The flow rate did not exceed 0.3 ml./min.

The neutral red indicator was adsorbed at the top of the column, where it appeared as an orange-coloured zone.

2 ml. 0.25 N HCl (0.5 m.equiv.) was carefully pipetted into the tube. The tap was opened and the flow adjusted to 0.2 ml./min. The acid was followed by 1.5 ml. water. The eluate was collected in a 10 ml. stoppered, graduated tube; the total volume was 3.6 ml. and contained 24 mg. Na^+ as NaCl (0.41 m.equiv.). As the *p*H fell, the column became shorter and contributed 0.1 ml. to the eluate.

Note on Stability.—The procedure could be interrupted without loss of histamine at the step preceding the adsorption or after the elution. The solutions were then stored at −17°. Control solutions containing known amounts of histamine (usually 50 ng.), and the solutions from which the histamine standards

were prepared, were also kept in the frozen state. Under these conditions the solutions retained their activity for months.

Reconstitution of the Eluate for Bioassay.—The final volume of the solution was 5 ml. and so provided 8 doses for the assay (using 0.6 ml./dose). The method has already been discussed and will only be summarized here.

1 drop of the neutral red indicator was added to the eluate and the free HCl neutralized with N NaOH (0.09 ml. ± 0.01 (25)), until the pink colour disappeared. The colour was brought back with 0.1 N HCl (0.02 to 0.05 ml.) and a further adjustment made, if necessary, with a trace of 0.1 N NaOH. The additional NaCl formed in back-titrating the NaOH could usually be neglected; if it exceeded 5% of the stoichmetric value (29.2 mg. NaCl), allowance was made in calculating the balance required to make the final solution isotonic. It was normally assumed that the neutralized eluate (3.69 ml.) contained 30 mg. NaCl. The following solutions were added: NaCl (80 g./l.), 0.125 ml. Solution A (which consists of a mixture of equal volumes of the following solutions in g./l.: KCl (8) + $CaCl_2$ (5.5) + $MgCl_2$ (4) + NaH_2PO_4 (0.05), 0.5 ml. $NaHCO_3$ (36.6 g./l.), 0.05 ml., and water to 5.00 ml. The sodium bicarbonate was added at the time when the histamine standards were prepared. The *p*H of the reconstituted solutions was 7.7 ± 0.2 (35) and differed only slightly from that of the Tyrode used (*p*H 7.7). The *p*H values were obtained from freshly made solutions.

Separation of Histamine by Adsorption on IRC-50 Columns

A 5 ml. aliquot of SN obtained after precipitating the proteins in 5 ml. plasma was neutralized with N NaOH (0.7 ml.), and diluted to 12 ml. with buffer *p*H 5.9. The final sodium ion concentration of the solution was calculated to be 0.21 N.

The solution for adsorption was applied to the column (prepared as directed above), which was afterwards washed with 5 ml. water. Elution was performed with 5 ml. 0.25 N HCl (1.25 m.equiv.), followed by 1.5 ml. water. The eluate was collected in a 10 ml. stoppered, graduated tube; the total volume was 6.7 ml. and contained 54 mg. NaCl. Shrinkage of the column contributed 0.2 ml. 1 drop of neutral red indicator was added and the remaining acid was neutralized with N NaOH (0.32 ml. ± 0.07 (25)). The total amount of NaCl was thus 73 mg. in 7.0 ml.; this solution was reconstituted to 10 ml. and assayed.

Assay and Identification of Histamine.—The assay was performed on the superfused guinea-pig ileum in comparison with histamine standards prepared in Tyrode solution containing atropine (100 ng./ml.).

At the end of the assays, Tyrode solution containing mepyramine maleate (2 to 4 ng./ml.) was allowed to flow over the intestine. The assays were repeated to test for active substances other than histamine. The concentration of histamine in the plasma was obtained by multiplying the result of the assay in ng./ml. by a factor depending on the volume of supernatant taken for the adsorption.

Six to eight plasma samples could be conveniently treated at one time. The method, including the assays, required two days.

Results

Recovery Experiments

The method was tested in a series of experiments in which histamine was added to solutions of known composition and to plasma.

Histamine Added to Solutions of Known Composition

Reconstituted Eluates Obtained from Blank Columns.—The effect of solutions from composite columns which had received only buffer or the reagents occasionally exceeded the zero effect of Tyrode solution; but it was always equivalent to less than 0.5 ng./ml. and often to less than 0.25 ng./ml. of the histamine standard. When histamine was added to give a concentration of 1 to 2.5 ng./ml. in the solution, the mean recovery was 101% ± 6 (10). In similar experiments with IRC-50 columns, the effect of the solution was usually greater than the zero effect of Tyrode solution. When histamine was added to give a concentration of 2 ng./ml., the mean recovery was 102% ± 15 (12).

Solutions Applied to Composite Columns (p*H 8*).—50 ng. histamine was added to solutions of the following composition: 2 ml. stock buffer (*p*H 7.92) + 0.77 ml. N NaCl + N NaTCA (0.5 to 3.0 ml.); the volume was made up to 14 ml. The solutions were applied to columns containing 50 mg. XE-64 which had been previously equilibrated with buffers (*p*H 8) of similar strength. In the range 0.2 N to 0.25 N, the recovery was 87% (2 estimations), at 0.35 N, 80%, and at 0.45 N, 72%. These results confirmed those previously obtained with buffer solutions and showed that the sensitivity of the columns to sodium ion concentration was slightly reduced by increasing the quantity of resin from 30 to 50 mg.

Solutions Applied to IRC-50 Columns (p*H 5.9*).—When 50 ng. histamine was added to 10 ml. of the stock buffer (*p*H 5.9; 0.23 N [Na^+]), the mean recovery was 89% ± 9 (10). Similarly, when 100 ng. was added to 25 ml. of the buffer the recovery was 95% (range (5) 85 to 100). Recoveries in this range were also obtained when 50 ng. was added to 25 ml. of a solution containing NaTCA (12 ml. 0.23 N NaTCA + 5 ml. stock buffer *p*H 5.9), and to Tyrode solution (5 ml.) diluted with stock buffer (5 ml.). It was apparent from these experiments

that IRC-50 could be used to adsorb histamine from volumes of solution greater than 20 ml., when the sodium ion concentration was approximately 0.2 N.

Potassium ions and the divalent cations present in Tyrode solution would be expected to exchange with sodium ion and to appear in the eluate. The extent to which this occurred was not investigated, since there was no evidence that the recoveries from Tyrode solution differed in any way from those obtained with buffer solution.

Histamine Added to Plasma

The results obtained with composite columns (*p*H 8) are contained in Table II. They are uncorrected for the effect of the plasma control, since

TABLE II

RECOVERY OF HISTAMINE ADDED TO PLASMA

Adsorption on composite column (XE-64) buffered at *p*H 8. Expt. Nos. 1 to 5, 5 ml. human plasma; expt. Nos. 6 and 7, 2.5 ml. cat plasma. Volume of reconstituted eluate 5 ml. The amounts of histamine are given in ng.

Expt. No.	Amount of Histamine Added			
	0	25	50	100
	Amount of Histamine Recovered			
		%	%	
1	<2·5	20 (80)	44 (88)	80
2	<2·5	15 (60)	40 (80)	84
3	<2·5	18 (72)	45 (90)	88
4	<2·5	20 (80)	45 (90)	90
5	3·0	21 (84)	45 (90)	90
6	<3·5	— —	44 (88)	—
7	<1·5	— —	— —	76
Mean		18·8 (75·2)	43·8 (87·6)	84·6

Mean % recovery in the range 25 to 100 ng. 82·5±2·0 S.E.

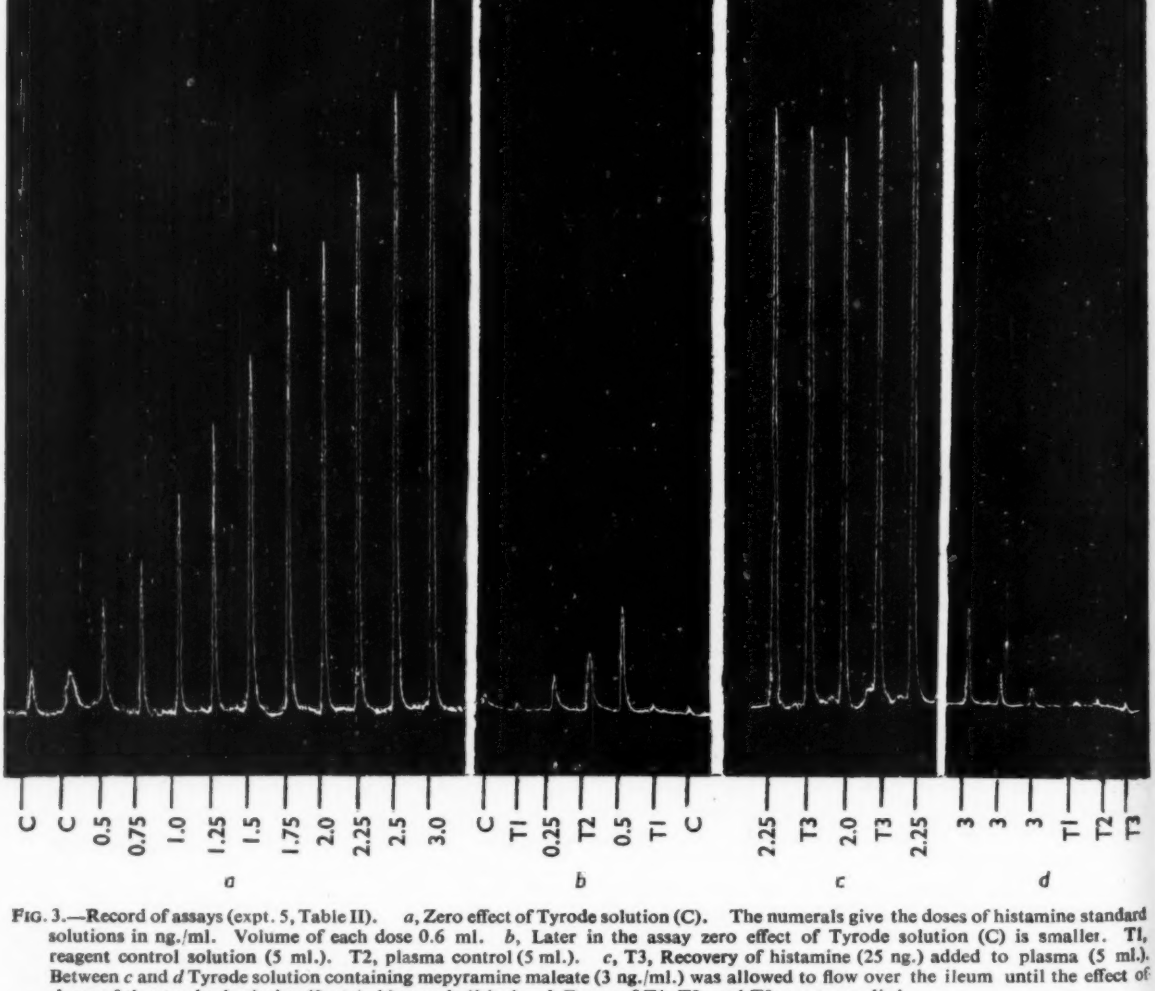

FIG. 3.—Record of assays (expt. 5, Table II). *a*, Zero effect of Tyrode solution (C). The numerals give the doses of histamine standard solutions in ng./ml. Volume of each dose 0.6 ml. *b*, Later in the assay zero effect of Tyrode solution (C) is smaller. T1, reagent control solution (5 ml.). T2, plasma control (5 ml.). *c*, T3, Recovery of histamine (25 ng.) added to plasma (5 ml.). Between *c* and *d* Tyrode solution containing mepyramine maleate (3 ng./ml.) was allowed to flow over the ileum until the effect of doses of the standard solution (3 ng./ml.) was abolished. *d*, Doses of T1, T2, and T3 were reapplied.

in six out of the seven experiments this had less activity than could be measured or was equal to the zero effect of the Tyrode solution or of the reagent control. Tracings obtained in experiment 5 are shown in Fig. 3. In this experiment the histamine equivalent of the control plasma was estimated to be 0.6 ng./ml. (uncorrected for the 10% dilution of the blood sample with saline). The mean recovery of histamine added in the range 25 to 100 ng. was 82.5% ± 2 S.E. Results obtained in earlier experiments by adsorption on IRC-50 columns (*p*H 5.9) are shown in Table III. The mean recovery (81.4%) did not differ significantly

TABLE III

RECOVERY OF HISTAMINE ADDED TO PLASMA

Adsorption on IRC-50 columns buffered at *p*H 5·9. Expt. Nos. 1 to 5, 5 ml. human plasma; expt. Nos. 6 and 7, 5 ml. cat plasma. Volume of reconstituted eluate 10 ml. The values for histamine are in ng.

Expt.	Amount of Histamine Added		
	0	50	
	Amount of Histamine Recovered		%
1	< 5	39	(78)
2	< 5	40	(80)
3	< 5	40	(80)
4	< 5	44	(80)
5	< 5	45	(90)
6	< 5	40	(80)
7	< 10	41	(82)
	Mean	40·7	81·4 ± 1·7 S.E.

(P>0.05) from the mean (87.6%) obtained with the same quantity of histamine applied to composite columns.

Histamine was also added in a concentration of 1 to 3 ng./ml. to reconstituted eluates of control plasma samples. The mean recovery was 98% ± 8 (15). The result shows that the final solution appeared to be free from substances in plasma which might interfere with the assay. This conclusion was strengthened by the results of experiments in which either adrenaline (50 ng.) or 5-HT (50 ng.) was added to plasma which was extracted by the final method. The reconstituted eluates (5 ml.) were devoid of activity on the guinea-pig ileum. When histamine was added to them in a concentration of 2 ng./ml., the recoveries fell within the same range. If the eluate had contained half of the adrenaline added to the plasma, the concentration (5 ng./ml.) would have reduced the response to histamine (2 ng./ml.) by about 10%. Similarly, if it had contained half of the 5-HT (5 ng./ml.), the response would have been increased by about 10%, and the effect of the 5-HT been detectable in the presence of mepyramine (4 ng./ml.).

Application of the Method.—The results of experiments in which histamine was administered by slow intravenous infusion to anaesthetized cats are shown in Table IV. In one experiment IRC-50 columns (*p*H 5.9) were used for the adsorption; in the other, composite columns buffered at *p*H 8 which made it possible to employ smaller volumes (2.5 ml.) of plasma without loss of sensitivity. Infusion at the rate of 330 ng./kg./min. produced no obvious fall in the arterial blood pressure, yet the plasma histamine rose to a measurable concentration in samples of arterial blood. The sample taken 10 min. after the infusion of histamine had stopped showed that the concentration of extractable histamine had fallen to the control value. The total dose infused in each experiment was 20 μg. in 20 min.

TABLE IV

ESTIMATION OF PLASMA HISTAMINE AFTER INTRAVENOUS INFUSION OF HISTAMINE IN THE CAT

Pentobarbitone sodium anaesthesia. Heparin 5 mg./kg.

Time (min.)	Infusion	Blood Sample (Femoral Artery) (ml.)	Haematocrit	Vol. Plasma Extracted (ml.)	Plasma Histamine (ng./ml.)
Experiment 1. Male Wt. 3.0 kg. Adsorption on composite column (*p*H 8)					
−5 to 0	0·9% NaCl	5	44	2·5	<0·3
0 to +15	Histamine 330 ng./kg./min. in 0·9% NaCl	5	43	2·5	3·1
+15 to +20	,,	5	42	2·5	3·7
+20 to +30	0·9% NaCl	5	42	2·5	<0·3
Experiment 2. Male Wt. 3·2 kg. Adsorption on IRC-50 column (*p*H 5·9) (Carotid Artery)					
−5 to 0	0·9% NaCl	10	—	4·0	1·6
0 to +15	Histamine 330 ng./kg./min. in 0·9% NaCl	10	—	4·5	5·6
+15 to +20	,,	10	—	4·0	5·5
+20 to +30	0·9% NaCl	10	—	4·0	<1·3

DISCUSSION

The method makes it possible to purify TCA extracts of plasma so that the bioassay can be performed by placing the final solution in direct contact with the suspended strip of guinea-pig ileum. It involves the assumption that the histamine extracted by the TCA is evenly distributed between the supernatant and the precipitate. The results of recovery experiments support this assumption. The purification achieved at the adsorption step can only be partial by chemical standards; nevertheless it appears to be sufficient for the method of bioassay used. The reconstitution

procedure makes use of the sodium ion displaced from the resin, and losses which would occur by evaporating the eluate and extracting the histamine with ethanol or in other ways are thereby avoided.

The purification depends as much on the initial treatment of the blood and plasma as on the subsequent procedures. Early in this work an attempt was made to adsorb histamine and acetylhistamine cations directly from plasma which had been diluted with buffer (*p*H 5.9). Evidence was obtained that the amines could be removed quantitatively in this way, but the results were complicated by the formation of a slow contracting substance which appeared in the acid eluate (Gaddum, 1956). A method was devised for the separation of histamine from this unidentified substance (X), but it proved to be too laborious for the routine estimation of histamine. It was therefore decided to estimate instead the extractable histamine after precipitation of the proteins with TCA. Substance X, which shares some of the properties of bradykinin (Rocha e Silva, Beraldo, and Rosenfeld, 1949), may have appeared because of contact between plasma and glass (Armstrong, Jepson, Keele, and Stewart, 1954) or resin, or because the plasma was diluted with buffer (Schachter, 1956). Careful treatment of the blood and plasma by methods similar to those employed by Armstrong *et al.* (1954) probably delayed the formation of substance X, so that when the plasma proteins were precipitated two to three hours after obtaining the blood samples, none was detectable in the solution for assay.

The blood was centrifuged at a low value of *g* to separate histamine contained in the cells. The plasma was centrifuged to remove 5-HT and histamine contained in the platelets. The concentration of platelets remaining in the centrifuged plasma was apparently not high enough to influence the result through the release of 5-HT or histamine or an unidentified plain-muscle stimulating substance (Humphrey and Jaques, 1954).

The quantity of resin chosen for the adsorption was limited, on the one hand, by the minimum needed for the complete removal of histamine from the solution, and, on the other, by the exchange capacity of the resin at the *p*H of the adsorption. The final volume of the test solution depended on the quantity of sodium released from the resin when this was treated with acid. For example, the exchange capacity of 325 mg. IRC-50 at *p*H 5.9 is approximately 0.9 m.equiv. Na^+ (or 54 mg. Na^+ as NaCl); the eluate must then be reconstituted to 10 ml. At *p*H 8, the capacity of the resin increases by nearly 3-fold and the final solution could not be less than 20 ml. It was possible to reduce the sodium, and hence the volume of the solution, by using the finely divided form of the resin. At *p*H 8.0, 50 mg. of the resin (XE-64) has a capacity of 0.4 m.equiv. Na^+ (or 24 mg. Na^+ as NaCl) which leaves a margin of 16 mg. NaCl for the final neutralization of the eluate and its reconstitution to 5 ml. The powdered cellulose mixed with the resin adds only slightly to the exchange capacity. However, the adsorption of histamine on composite columns may be incomplete if the solution for adsorption contains too much sodium ion; it is important that the quantity of Na^+ as NaTCA in the solution (10 ml.) should not exceed 1 m.equiv. (185 mg.). This limits the volume of plasma that can be extracted by the method as described to 5 ml. or less.

Adsorption at *p*H 8 makes it possible to estimate acetylhistamine independently; it may also provide better purification of the histamine than could be obtained at a lower *p*H, since pharmacologically active bases that are weaker than histamine would be less strongly adsorbed. Adrenaline ($pK\alpha$ 10.7 [cation]; Albert, 1954) and 5-HT ($pK\alpha$ 9.1; Rapport, Green, and Page, 1948) are probably adsorbed at *p*H 8, but they are unstable in an alkaline medium and so lose pharmacological activity. Thus at *p*H 8, 50 ng. of adrenaline in pure solution may be expected to become inactive in less than 30 min. (Dr. T. B. B. Crawford, personal communication) and 50 ng. 5-HT to lose more than 20% of its activity in the same time (Rapport *et al.*, 1948).

IRC-50 columns buffered at *p*H 5.9 have the disadvantage that the volume of the reconstituted eluate is large (10 ml.). When small volumes of plasma are extracted (2.5 ml.), the histamine contained in the aliquot of the SN (3 ml.) is diluted 3.3-fold and may not be detectable at the assay. Further, adrenaline and 5-HT are stable at *p*H 5.9 and will therefore be adsorbed and eluted. Nevertheless, the recoveries at this *p*H indicate that these bases were not present in the plasma in sufficient quantity to interfere with the recovery of histamine when the assay was performed on the superfused guinea-pig ileum.

The quantity of histamine extractable from plasma obtained from man and the cat was in all instances less than 1 ng./ml. This value is lower than most of the published results (Code, 1952). Thus Emmelin (1945) estimated histamine in ultrafiltrates and in TCA extracts (Code, 1937a) of plasma obtained from the cat and found values in the range 25 to 50 ng./ml. Born and Vane (1952) estimated the freely diffusible histamine in cat blood and obtained similar results (9 to 55 ng./ml.)

The low values for human plasma agree with those previously obtained by a different method (Adam, Card, Riddell, Roberts, and Strong, 1954) and with those reported by Lowry, Graham, Harris, Priebat, Marks, and Bregman (1954) and by Code (1937b). Lowry *et al.* (1954) determined the concentration of histamine in human plasma by a sensitive chemical method and found it to be less than 3 ng./ml. Code (1937b) found that when he protected the blood against trauma and did not use anticoagulants, the plasma histamine in man was less than could be detected by his method of assay (probably 20 ng./ml. plasma). High values for the plasma histamine may be due to damage of the blood or to the liberation of histamine from pharmacologically inactive forms. It is uncertain whether or not human plasma contains detectable quantities of acetylhistamine. This would in any case not be hydrolysed by the TCA used in the extraction (Dr. V. R. Pickles, personal communication). However, when heparin is used as an anticoagulant and the TCA extract is boiled in strong HCl, there is evidence to suggest that some of the histamine activity derives from the heparin (unpublished results). Impurities in the TCA extract (Code, 1937a) may also contribute to the activity of the test solution (Mongar and Whelan, 1953).

The results of the infusion experiments suggest that the present method might be as sensitive as that of the gastric secretion for detecting histamine in the plasma of anaesthetized cats. According to Emmelin, Kahlson, and Wicksell (1941) and Wood (1948), the rate of infusion for a submaximal effect on the gastric secretion varied from 700 to 1,500 ng./kg./min., which is still 10 to 20 times higher than the rate required for a submaximal effect on the gastric secretion in the conscious dog (Hanson, Grossman, and Ivy, 1948) or man (Adam, Card, Riddell, Roberts, Strong, and Woolf, 1954).

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References

Adam, H. M., Hardwick, D. C., and Spencer, K. E. V. (1954). *Brit. J. Pharmacol.*, **9**, 360.
—— Card, W. I., Riddell, M. J., Roberts, M., and Strong, J. A. (1954). Ibid., **9**, 62.
—— —— —— —— —— and Woolf, B. (1954). Ibid., **9**, 329.
Albert, A. (1954). *Pharmacol. Rev.*, **6**, 143.
Armstrong, D., Jepson, J. B., Keele, C. A., and Stewart, J. W. (1954). *Nature, Lond.*, **174**, 791.
Barsoum, G., and Gaddum, J. H. (1935). *J. Physiol.*, **85**, 1.
Bergström, S., and Hansson, G. (1951). *Acta physiol. scand.*, **22**, 86.
Born, G. V. R., and Vane, J. R. (1952). *Brit. J. Pharmacol.*, **7**, 298.
Bregman, J. I. (1953). *Ann. N.Y. Acad. Sci.*, **57**, Art 3, 125.
Code, C. F. (1937a). *J. Physiol.*, **89**, 257.
—— (1937b). Ibid., **90**, 349.
—— (1952). *Physiol. Rev.*, **32**, 47.
—— and McIntire, F. C. (1956). *Methods of Biochemical Analysis*, vol. 3, p. 49. Ed. D. Glick. London: Interscience.
Condon, N. E. (1954). *Science Technologists Bulletin*, **3**, 9.
Emmelin, N. (1945). *Acta physiol. scand.* **11**, Suppl. 34, 37.
—— Kahlson, G. S., and Wicksell, F. (1941). *Acta physiol. scand.*, **2**, 123.
Gaddum, J. H. (1953). *Brit. J. Pharmacol.*, **8**, 321.
—— (1956). *Ciba Foundation Symposium on Histamine*, p. 43. Ed. G. E. W. Wolstenholme and C. M. O'Connor. London: Churchill.
Hanson, M. E., Grossman, M. I., and Ivy, A. C. (1948). *Amer. J. Physiol.*, **153**, 242.
Humphrey, J. H., and Jaques, R. (1954). *J. Physiol.*, **124**, 305.
Kunin, R., and Meyers, R. J. (1950). *Ion Exchange Resins*, p. 26. New York: John Wiley.
Levy, M. (1935). *J. biol. Chem.*, **109**, 361.
Lowry, O. H., Graham, H. T., Harris, F. B., Priebat, M. K., Marks, A. R., and Bregman, R. V. (1954). *J. Pharmacol.*, **112**, 116.
Mongar, J. H., and Schild, H. O. (1950). *J. Physiol.*, **111**, 47P.
—— and Whelan, R. F. (1953). Ibid., **120**, 146.
Partridge, S. M. (1954). *Soc. of Chem. Indust., Congr. on Ion Exchange and its Applications*, Proceedings, p. 117.
Rapport, M. M., Green, A. A., and Page, I. H. (1948). *J. biol. Chem.*, **176**, 1237, 1243.
Roberts, M., and Adam, H. M. (1950). *Brit. J. Pharmacol.*, **5**, 526.
Rocha e Silva, M., Beraldo, W. T., and Rosenfeld, G. (1949). *Amer. J. Physiol.*, **156**, 261.
Samuelson, O. (1952). *Ion Exchangers in Analytical Chemistry*, p. 9. New York: John Wiley.
Schachter, M. (1956). *Brit. J. Pharmacol.*, **11**, 111.
Tabor, H., and Mosettig, E. (1949). *J. biol. Chem.*, **180**, 703.
Wood, D. R. (1948). *Brit. J. Pharmacol.*, **3**, 231.

Brit. J. Pharmacol. (1957), **12**, 406.

THE EFFECT OF LYSERGIC ACID DIETHYLAMIDE, 5-HYDROXYTRYPTAMINE, AND RELATED COMPOUNDS ON THE LIVER FLUKE, *FASCIOLA HEPATICA*

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The rhythmical activity of the liver fluke, *Fasciola hepatica*, was stimulated by 5-hydroxytryptamine and by lysergic acid diethylamide at very low concentrations. The effect was peripheral and was not mediated through the central ganglion. Other amines also stimulated rhythmical activity, the most potent being the indolamines.

Bromolysergic acid diethylamide, and other analogues such as yohimbine, harmine, and dopamine depressed rhythmical movement and antagonized the stimulant action of 5-hydroxytryptamine and lysergic acid diethylamide. Evidence which suggests the presence of tryptamine receptors in the trematode is discussed.

In a previous investigation it was reported by Chance and Mansour (1949) that the liver fluke, *Fasciola hepatica*, can be used as a preparation for studying the effect of drugs on trematodes. It was found that the tone, rate, and amplitude of muscular contractions of the parasite were stimulated by amphetamine. Recent investigations by Welsh (1953) suggest that 5-hydroxytryptamine (5-HT) is a mediator of nerve action in certain invertebrates. Erspamer (1954) demonstrated the presence of 5-HT and related indolalkylamines in many invertebrate tissues. In view of these recent developments and in an attempt to obtain a better understanding of muscular activity of trematodes, it was decided to test the effect of 5-HT and of related amines on the activity of the liver fluke. In addition, observations were made upon the effect of lysergic acid derivatives on the movements of the parasite.

METHODS

The method of collecting and keeping liver flukes for the study of their movement has been described in a previous paper (Chance and Mansour, 1949). In addition to recording the movements of the intact parasite on a kymograph, a nerve muscle preparation was made in the following way. The oral end of the parasite in front of the posterior sucker was cut off, thus eliminating the effects of the circumoesophageal ganglion. The remainder of the body was then divided longitudinally into two halves. Each half was attached to a recording lever writing on a kymograph in the same way as the intact parasite preparation. The tension of the lever was 0.3 g. The preparations were completed as quickly as possible in a glass petri dish with a waxed bottom, containing Ringer solution at 37°. When mounted in a 10 ml. organ bath at 38°, these preparations did not show rhythmical activity. They will be referred to as "nerve-muscle" preparations in the text because each one consists of two layers of exposed muscle without any ganglionic control. Recording was stopped during addition of a drug to the organ bath. After addition of the drug in 0.1 ml. of solution or less, the medium was stirred by bubbling air for 1 min. to ensure uniform distribution of the compound. Solutions of drugs were adjusted to the *p*H of the medium before addition. Drugs were tested initially at a high concentration and, if active, the minimum effective concentration was determined with at least 4 preparations. The composition of the Ringer solution used was NaCl 9.0 g., KCl 0.4 g., $CaCl_2$ 0.06 g., $NaHCO_3$ 0.5 g., glucose 0.5 g./l.

RESULTS

Effect of Lysergic Acid Diethylamide and 5-Hydroxytryptamine.—Both lysergic acid diethylamide (LSD) and 5-HT stimulated the rhythmical activity of the liver fluke preparations. Concentrations of LSD higher than 10^{-7} M produced a transient increase in the tone and a great increase in amplitude of rhythmical movement of the intact parasite (Fig. 1*a*). When added to the nerve-muscle preparations at concentrations higher than 10^{-7} M, LSD initiated contractions of high ampli-

tude (Fig. 1*c*). It was observed that LSD at concentrations of 5×10^{-8} to 5×10^{-9} M caused a decrease in the rate, amplitude, and tone. This effect was followed by an increase in tone (Fig. 1*b*). The minimum concentration of 5-HT which produced stimulation was 5×10^{-5} M for the intact parasite preparation and 2.5×10^{-5} M for the nerve-muscle preparation. The stimulant effect of 5-HT on intact parasite preparations was characterized by an increase of tone, rate, and amplitude (Fig. 2). The degree of stimulation was greater in intact parasite preparations which showed weak spontaneous rhythmical movement. The response of the nerve-muscle preparation to both LSD and to 5-HT indicates that stimulation can occur in the absence of the central ganglion of the parasite.

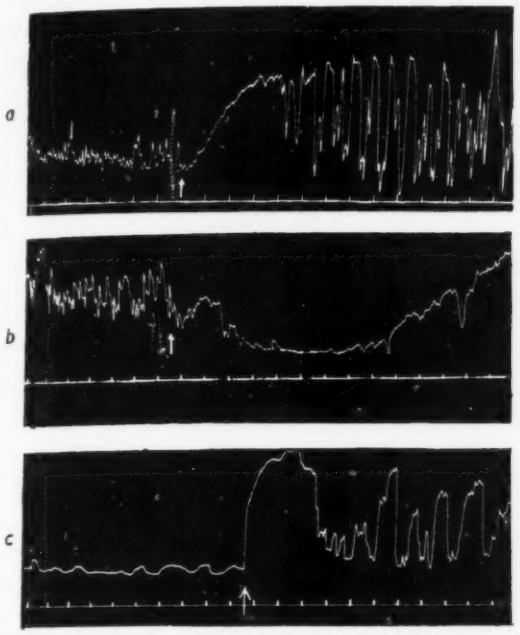

FIG. 1.—Liver fluke. Effect of lysergic acid diethylamide. In all tracings the upward stroke represents contraction recorded on a kymograph. Time markings are in min. The drugs were added at the points indicated by the arrows. (*a*) LSD, 10^{-7} M, on intact parasite preparation, (*b*) LSD $.5 \times 10^{-9}$ M, on intact parasite preparation, (*c*) LSD, 2×10^{-7} M, on nerve-muscle preparation.

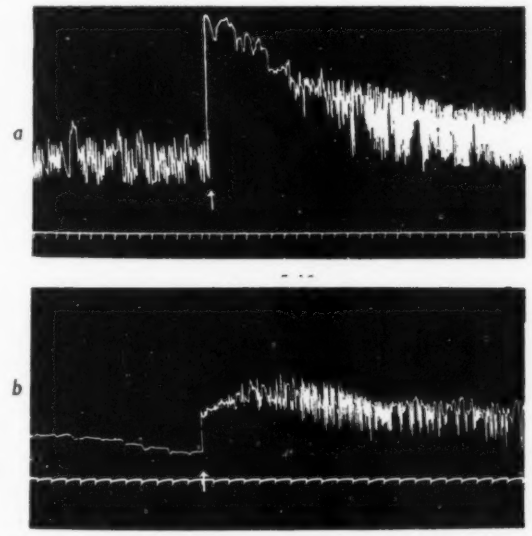

FIG. 2.—Liver fluke. Recordings as in Fig. 1. Effect of 5-hydroxytryptamine, 5×10^{-4} M, (*a*) on intact parasite preparation, (*b*) on nerve-muscle preparation.

Stimulant Effect of Other Amines.—Other indole derivatives and amines were tested on both preparations in order to observe any correlation between chemical structure and stimulant activity. Substances listed in Table I stimulated the intact parasite as well as the nerve-muscle preparation, indicating that the stimulant effect is peripheral and is not mediated by the central ganglion. The stimulant effect of these compounds was similar to that produced by 5-HT. Among the indolamines, 5-HT stimulated the preparations at a lower molar concentration than tryptamine, bufotenine, or 1-benzyl-5-methoxy-2-methyltryptamine. 5-Hydroxytryptophan stimulated both preparations less than did the indolamines. The stimulant effect of this compound was observed only after 5 to 10 min. Possibly the stimulant effect of 5-hydroxytryptophan is caused by a metabolic product rather than by the drug itself. The benzylamines, amphetamine, ephedrine, mephentermine, and mescaline, stimulated both the intact parasite and the nerve-muscle preparation, but at higher concentrations than did the indolamines.

TABLE I
POTENCY OF STIMULANT COMPOUNDS

Compound	Min. Effective Molar Conc.	
	Intact Prep.	Nerve-muscle Prep.
Lysergic acid diethylamide ..	10^{-7}	10^{-7}
5-Hydroxytryptamine ..	$5{\cdot}0 \times 10^{-5}$	$2{\cdot}5 \times 10^{-5}$
Tryptamine	$5{\cdot}0 \times 10^{-4}$	5×10^{-4}
Bufotenine	$2{\cdot}5 \times 10^{-4}$	5×10^{-4}
1-Benzyl-5-methoxy-2-methyltryptamine	10^{-4}	10^{-4}
5-Hydroxytryptophan ..	4×10^{-3}	4×10^{-3}
Amphetamine	3×10^{-4}	$1{\cdot}5 \times 10^{-4}$
N-Methylphenyltertiary butylamine (mephentermine) ..	6×10^{-4}	6×10^{-4}
Ephedrine	10^{-3}	10^{-3}
3: 4: 5-Trimethoxyphenylethylamine (mescaline)	5×10^{-3}	5×10^{-4}

Drugs which Inhibited Rhythmical Activity.—Substances listed in Table II depressed the rhythmical activity of the intact parasite preparations

TABLE II

DRUGS WHICH DEPRESSED RHYTHMICAL ACTIVITY OF INTACT PARASITE PREPARATIONS

Drugs	Min. Effective Molar Conc.
Bromolysergic acid diethylamide ..	10^{-7}
Yohimbine	10^{-4}
Harmine	5×10^{-4}
3: 4-Dihydroxyphenylethylamine (dopamine)	10^{-3}

sometimes to the point of complete paralysis. These compounds did not have any effect on the nerve-muscle preparations. Bromolysergic acid diethylamide (BOL) was the most potent drug, depressing the intact parasite preparation at concentrations above 10^{-7} M (Fig. 3). Yohimbine at

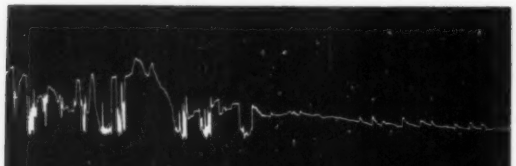

FIG. 3.—Liver fluke. Recording as in Fig. 1. Effect of bromolysergic acid diethylamide, 10^{-6} M, on intact parasite preparation.

concentrations higher than 10^{-4} M and harmine at concentrations higher than 5×10^{-4} M depressed rhythmical activity. 3:4-Dihydroxyphenylethylamine (dopamine), which has been shown to be rapidly oxidized by a phenol oxidase in these parasites (Mansour, 1957), inhibited rhythmical activity at concentrations higher than 10^{-3} M.

Antagonistic Effect of Stimulatory and Inhibitory Drugs.—The effects of 5-HT, LSD, and amphetamine were tested on both preparations in the presence of various concentrations of BOL. It was found that BOL antagonized the effect of these stimulants. Furthermore, the depressant effect of BOL on the intact parasite preparation did not occur in the presence of the stimulant drugs (Figs. 4 and 5). Table III gives the concentrations of BOL and various stimulant drugs which, when added together, did not affect significantly the motility of either preparation. When the concentration of the stimulant was increased, it was necessary to increase the concentration of the antagonist to overcome the stimulation. For example, BOL in a concentration of 10^{-6} M antag-

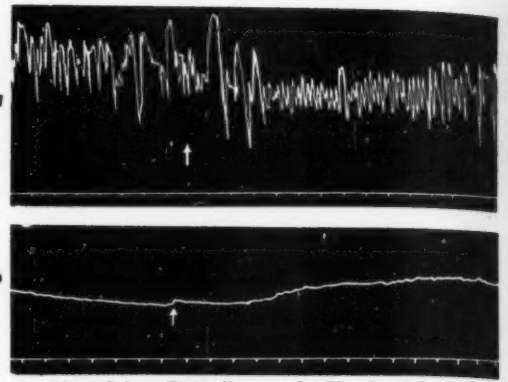

FIG. 4.—Liver fluke. Recordings as in Fig. 1. Effect of bromolysergic acid diethylamide, 10^{-7} M, added together with lysergic acid diethylamide, 10^{-7} M, (*a*) on intact parasite preparation, (*b*) on nerve-muscle preparation.

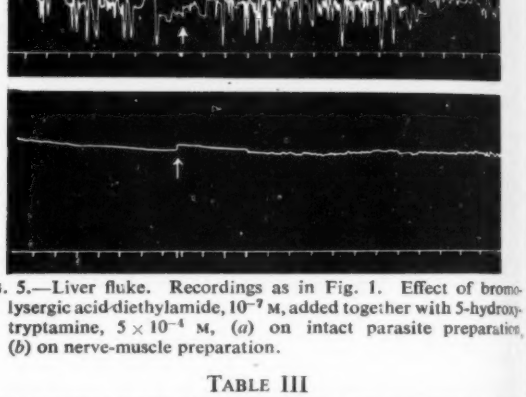

FIG. 5.—Liver fluke. Recordings as in Fig. 1. Effect of bromolysergic acid diethylamide, 10^{-7} M, added together with 5-hydroxytryptamine, 5×10^{-4} M, (*a*) on intact parasite preparation, (*b*) on nerve-muscle preparation.

TABLE III

ANTAGONISM OF BROMOLYSERGIC ACID DIETHYLAMIDE TO STIMULANT DRUGS

The antagonistic BOL concentrations were determined with at least 4 preparations.

Stimulant Drug (Molar Conc.)	Antagonistic BOL Molar Conc.	
	Intact Prep.	Nerve Muscle Prep.
5-Hydroxytryptamine 5×10^{-4}	10^{-6}	10^{-5} to 10^{-4}
,, 10^{-3}	10^{-5}	10^{-4}
Lysergic acid diethylamide 10^{-7}	10^{-7}	10^{-7}
,, ,, ,, 10^{-6}	10^{-6}	10^{-6}
Amphetamine 5×10^{-4} ..	10^{-6}	10^{-6}
,, 10^{-3} ..	10^{-5}	10^{-5}

onized the stimulant effect of 5×10^{-4} M 5-HT. The concentration of BOL required to antagonize 10^{-3} M 5-HT was 10^{-5} M. The same phenomenon was observed when LSD or amphetamine was used as a stimulating agent. The concentration of BOL required to antagonize amphetamine was the same as that which antagonized 5-HT. Yohimbine

(10^{-4}) inhibited the stimulant effect of 5×10^{-4} M 5-HT on the intact preparation and 10^{-4} M on the nerve-muscle preparation. Dopamine (10^{-4} M) inhibited the stimulatory effect of 10^{-4} M 5-HT on both the intact and the nerve-muscle preparations. Harmine (5×10^{-4} M) did not interfere with the stimulant effect of 5-HT on either preparation.

Ineffective Compounds.—(−)-Adrenaline, (−)-noradrenaline, histamine, chlorpromazine, and sodium bromide at concentrations of 5×10^{-3} M had no effect on either preparation. The absence of any effect of sodium bromide indicates that the bromide ion does not interfere with rhythmical movement.

Discussion

The present study has shown that 5-HT stimulates muscular contraction of the trematode *Fasciola hepatica*. Furthermore, rhythmical movement in isolated muscle strips of the parasite was initiated by 5-HT, which indicates that the action of this substance is mediated by peripheral receptors. The powerful stimulant activity of 5-HT on these helminths and the fact that 5-HT occurs normally in many invertebrates (Erspamer, 1954) raise the possibility that this or a related compound may be the humoral transmitter for the peripheral receptors of *Fasciola*. Other chemical mediators such as histamine, adrenaline, or noradrenaline do not have any effect on the parasite.

Movement in the intact liver fluke can be depressed by BOL, harmine, and yohimbine, all of which contain the indole structure and may be looked upon as complex analogues of 5-HT. Possibly the depressant compounds act by combining with tryptamine receptors, thus blocking the effect of endogenously released transmitter. This hypothesis is supported by the fact that BOL antagonized the stimulant effects of 5-HT, LSD, and amphetamine on intact preparations. The stimulant effect of these compounds on nerve-muscle preparations was also inhibited by BOL, which, by itself, had no effect. Furthermore, it was found necessary to increase the concentration of the stimulant whenever the concentration of the depressant drug was increased. The similarity between the effects of amphetamine and 5-HT and the fact that BOL antagonized both compounds suggest that these amines may act on the same receptors. The fact that LSD inhibited rhythmical activity of intact parasite preparations at concentrations lower than its stimulant potency cannot yet be explained.

It is of interest to note that LSD acted on muscular activity in the same manner as 5-HT. Both compounds affect the metabolism of the liver fluke in a similar manner (Mansour, 1956). Recent reports by Shaw and Woolley (1956), and Welsh and McCoy (1957), indicate that LSD has activity similar to that of 5-HT in many vertebrate as well as invertebrate tissues. In the instance of *Fasciola hepatica*, LSD, on a molar basis, was a much more potent stimulant than 5-HT itself: this may be due to a greater affinity of the receptors for LSD. Certain analogues have been found to be potent antagonists of 5-HT and of LSD. Sollero, Page, and Salmoiraghi (1956) have recently demonstrated that BOL is a very potent antagonist to 5-HT on sensitized rat uterus. Ginzel and Mayer-Gross (1956) have made the observation that BOL inhibits LSD psychosis in man. The inhibitory effect of BOL to the action of LSD and of 5-HT in *Fasciola* appears to parallel its action on mammals. These findings support the hypothesis that the pharmacological effect of LSD and BOL could be attributed to the fact that these compounds are structural analogues of 5-HT.

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References

Chance, M. R. A., and Mansour, T. E. (1949). *Brit. J. Pharmacol.*, **4**, 7.
Erspamer, V. (1954). *Pharmacol. Rev.*, **6**, 425.
Ginzel, K. H., and Mayer-Gross, W. (1956). *Nature, Lond.*, **178**, 210.
Mansour, T. E. (1956). *Fed. Proc.*, **15**, 454.
—— (1957). *J. Pharmacol.*, **119**, 164.
Shaw, E., and Woolley, D. W. (1956). *Science*, **124**, 121.
Sollero, L., Page, I. H., and Salmoiraghi, J. (1956). *J. Pharmacol.*, **117**, 10.
Welsh, J. H. (1953). *Anat. Rec.*, **117**, 637.
—— and McCoy, A. C. (1957). *Science*, **125**, 348.

Brit. J. Pharmacol. (1957), **12**, 410.

THE ACTION OF 5-HYDROXYTRYPTAMINE AND SOME OF ITS ANTAGONISTS ON THE UMBILICAL VESSELS OF THE HUMAN PLACENTA

BY

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The vasoconstrictor action of 5-hydroxytryptamine (5-HT) in the human placental preparation is about 10 times stronger than that of adrenaline and is antagonized by anti-adrenaline compounds like phentolamine. Both 5-HT and adrenaline are antagonized by yohimbine and chlorpromazine. Specific and strong anti-5-HT action is demonstrated for lysergic acid diethylamide (LSD) and tryptamine. Both LSD and tryptamine in larger doses have a vasoconstrictor action. Mescaline has no certain modifying effect on the action of 5-HT, but itself causes vasoconstriction in large doses. The antihistamine drug phenbenzamine in histamine blocking doses abolishes the action of 5-HT in half the preparations tested. The ganglionic blocking agent trimetaphan in large doses antagonizes the action of 5-HT added subsequently, and also, to a lesser degree, the effect of adrenaline. Hexamethonium and tetraethylammonium bromides are ineffective in this preparation. No certain modifying action of reserpine on subsequently added 5-HT could be demonstrated, and the same was true for heparin even in very high concentrations.

5-Hydroxytryptamine (5-HT) continues to attract considerable attention as a possible factor in different experimentally produced psychotic conditions in animals and as a compound possessing vascular effects. Although 5-HT has rather weak and inconsistent effects upon the systemic blood pressure in different animals and in man (Erspamer, 1954), evidence is accumulating for its strong vasoconstrictor action in certain local vascular beds.

In a previous report from this laboratory (Eliasson and Åström, 1955), the pharmacological actions of a series of compounds were studied on the blood vessels of isolated human placentas. 5-HT was included in this series, and its actions were compared with those of adrenaline and noradrenaline. It was shown that 5-HT, in the same manner as the catechol derivatives, caused a constriction of the umbilical vessels and its potency in this respect was about 10 times that of adrenaline. 5-HT showed many pharmacological similarities to adrenaline and noradrenaline; thus its action in this preparation was abolished by dihydroergotamine and enhanced by cocaine. The same ratio between the potency of 5-HT and adrenaline has been demonstrated in the lung vessels of the dog and the cat by Ginzel and Kottegoda (1953).

In the experiments here the action of 5-HT on the placenta has been studied further and special consideration has been given to a series of drugs known to antagonize the actions of 5-HT in other preparations. Since the umbilical vessels do not receive any nerve supply (Schmitt, 1922; Guarna, 1934) it was felt that a study of 5-HT antagonists in this preparation might yield information of interest concerning the pharmacological mechanisms involved.

METHODS

Human placentae were obtained from the hospital as soon as possible after delivery. The vessels of the umbilical cord were dissected free and cannulae were inserted and tied in the vein and in one of the anastomosing arteries about 2 to 3 cm. from the insertion of the cord at the surface of the placenta. The organ was placed in Ringer solution at 37°. Perfusion by means of a Dale-Schuster pump was performed with Tyrode solution to which, in half the preparations, 15% (v/v) of a solution of 6% dextran and 0.9% NaCl (Pharmacia) had been added. The presence of dextran in the perfusion liquid was effective in preventing oedema formation but did not influence the pharmacological effects of the drugs tested. The perfusion fluid was aerated with 6.5% carbon dioxide in oxygen. The *p*H of the perfusion liquid measured on the outflow side was 7.3 to 7.4.

The pump frequency was set at 46 strokes/min. and the stroke volume at the beginning of an experiment

was adjusted so as to yield a perfusion pressure of 60 to 80 mm. Hg. The stroke volume invariably had to be increased during the following 15 to 30 min. as more and more of the vessels opened up. After 20 to 30 min., the sensitivity of the preparation towards adrenaline was tested, and, if good and reproducible results were obtained, the tests were commenced. From the beginning of the tests, the stroke volume of the pump was kept constant. The venous outflow was not reperfused and was led off by a rubber tube, the outlet end of which was level with the placenta. The flow observed ranged from 20 to 40 ml./min. The inflow pressure was measured and recorded by a mercury manometer. Since the output from the pump was kept constant during the determination period, increases in resistance (vasoconstriction) in the placenta could be recorded as pressure changes.

The placenta preparation showed considerable variation in sensitivity. Not only was this true for different preparations but also for the same preparation during the course of one experiment (Eliasson and Åström, 1955). A total of 81 preparations were perfused, and of these 33 had to be discarded for quantitative comparisons since they were either too insensitive or gave varying responses to repeated tests with constant doses of adrenaline.

Of the substances used the following were kindly supplied by pharmaceutical manufacturers: chlorpromazine and hexamethonium bromide, May and Baker; dextran, Pharmacia; heparin, Vitrum; 5-hydroxytryptamine creatinine sulphate (5-HT), Abbott; lysergic acid diethylamide tartrate (LSD), Sandoz; phenbenzamine (Lergitin), Recip; phentolamine (Regitin) and reserpine, Ciba; tetraethylammonium bromide, Astra; trimetaphan (Arfonad), Hoffman-La Roche.

Results

The Action of 5-HT.—The action of 5-HT on the umbilical vessels is usually purely constrictor. However, in about 1 out of 5 preparations a diphasic response can be observed. In such preparations the pressure, after an initial rise, shows a secondary fall below control level (Fig. 4A). This effect is commonly present only at the beginning of an experiment and is also sometimes observed, although less frequently, with adrenaline and noradrenaline under similar conditions.

The vasoconstrictor action of 5-HT is very strong and doses of 5 to 10 μg. often cause complete closure of the vessels. In doses that give a moderate pressor response (0.5 to 3 μg.), 5-HT does not alter the effect of adrenaline added subsequently.

Repeated doses of 5-HT in the placenta preparation usually produce the same pressor effect if the intervals between injections are long enough to permit a complete return of the pressure to the original level. However, in 2 out of 8 preparations in which this phenomenon was particularly studied, the effect of subsequent doses was slightly less than that of the first dose. In both these preparations a longer time was required for recovery than in the other 6 experiments; this was true also after test doses of adrenaline.

Tryptamine.—In doses of 2.5 to 10 μg., which were 2 to 5 times greater than the dose of 5-HT which produced a good pressor response, tryptamine caused no effect on the perfusion pressure. These doses sufficed, however, to abolish completely the action of 5-HT added subsequently (Fig. 1). The effect of adrenaline after the tryptamine administration was usually unchanged,

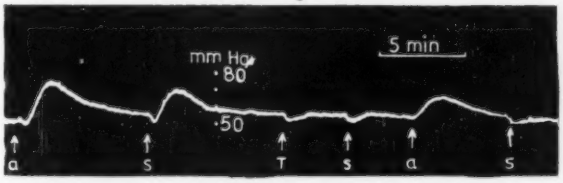

FIG. 1.—Record of umbilical arterial pressure in the isolated perfused human placenta showing the antagonistic action of tryptamine on 5-HT. The effect of adrenaline was slightly decreased after tryptamine in this preparation. a=adrenaline, 10 μg.; s=5-HT, 1 μg.; T=tryptamine, 5 μg.

although a slight decrease, as in Fig. 1, was observed in 2 out of 6 preparations. The histamine effect was unaffected by tryptamine. The preparation remained insensitive to 5-HT for a fairly short time, and normal responses could again be obtained 15 to 30 min. after the tryptamine administration.

Larger doses of tryptamine caused vasoconstriction. The activity in this respect was 10 to 20 times less than that of 5-HT.

Lysergic Acid Diethylamide.—LSD in doses of 5 to 25 μg. (1 to 5 times larger than the dose of 5-HT required to produce a good pressor response) had a constrictor action on the umbilical vessels in 2 out of 5 preparations. Whether LSD alone had any effect upon the perfusion pressure or not, the doses used always completely abolished the action of 5-HT. In 2 preparations the action of 5-HT was actually reversed after LSD. Administration of adrenaline and histamine before and after LSD gave identical results and thus no general decrease of sensitivity of the placenta preparation was caused by the LSD injections. The antagonistic effect of LSD against 5-HT is shown in Fig. 2. In this preparation the vasoconstrictor action of LSD is also illustrated.

Chlorpromazine.—This drug was given in doses of 0.5 to 1 mg. The action of 5-HT and of adrenaline was tested before the administration of

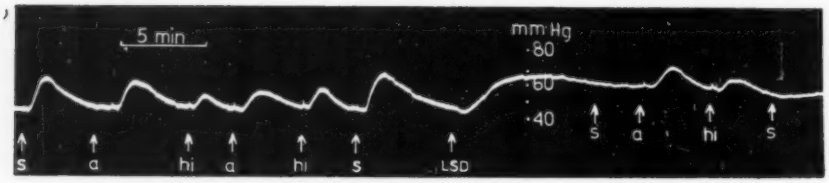

FIG. 2.—Record of umbilical arterial pressure in the isolated perfused human placenta. LSD (25 μg.) completely abolished the action of 5-HT (s, 5 μg.). The effects of adrenaline (a, 20 μg.) and histamine (hi, 25 μg.) were not significantly altered after LSD.

chlorpromazine and at different times afterwards. In the doses used, chlorpromazine completely abolished the effect of 5-HT and adrenaline. The antagonistic effect of chlorpromazine developed gradually and became maximal after 10 to 15 min. The 5-HT effect reappeared after about 30 min., but the action of adrenaline did not reappear during a further hour, after which time the preparation was usually discarded.

In 2 of 3 successful preparations, chlorpromazine in larger doses (2 to 2.5 mg.) had a constrictor action of its own. This effect could still be elicited even if the preparation had been rendered completely insensitive to adrenaline and 5-HT by previous administration of chlorpromazine.

Yohimbine.—When added to the umbilical circulation in doses of 20 to 100 μg. (20 to 50 times the dose of 5-HT required for a good response) yohimbine antagonized the action of subsequently added 5-HT and adrenaline. The anti-5-HT effect of yohimbine was more pronounced than its anti-adrenaline action (Fig. 3).

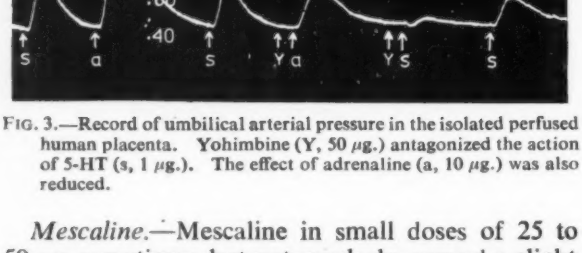

FIG. 3.—Record of umbilical arterial pressure in the isolated perfused human placenta. Yohimbine (Y, 50 μg.) antagonized the action of 5-HT (s, 1 μg.). The effect of adrenaline (a, 10 μg.) was also reduced.

Mescaline.—Mescaline in small doses of 25 to 50 μg. sometimes, but not regularly, caused a slight enhancement of the action of 5-HT. Larger doses from 50 to 300 μg. always had a vasoconstrictor effect. After these large doses the action of 5-HT as well as adrenaline was usually unaffected.

Phentolamine.—In the human placenta 1 to 3 mg. of phentolamine was found to abolish the action of both 5-HT and adrenaline.

Phenbenzamine.—This drug was selected as a representative of antihistamine drugs, and its influence upon the 5-HT effect was studied in 4 placenta preparations. Doses of phenbenzamine (30 to 50 μg.) which completely abolished the action of histamine in 2 of the 4 experiments also completely abolished the effect of 5-HT. In the other 2 experiments the action of 5-HT was not significantly altered after these small doses, which were nevertheless sufficient for histamine blockade. Tests with adrenaline before and after the administration of phenbenzamine were used to confirm that no general decrease of sensitivity had occurred during the experiment.

Reserpine.—The action of reserpine was studied in 4 preparations. Doses of 0.1 to 1.5 mg. had no effect on the perfusion pressure, nor could any certain modifying action on 5-HT be determined. The action of adrenaline also remained unchanged after the addition of reserpine.

Ganglionic Blocking Agents.—The action of tetraethylammonium bromide and hexamethonium bromide was studied in 10 preparations. Both substances were completely inactive in themselves and did not modify the action of 5-HT or adrenaline. The doses used ranged from 1 to 10 mg. for tetraethylammonium and from 1 to 25 mg. for hexamethonium and did not show any toxic effects in these experiments (Fig. 4A).

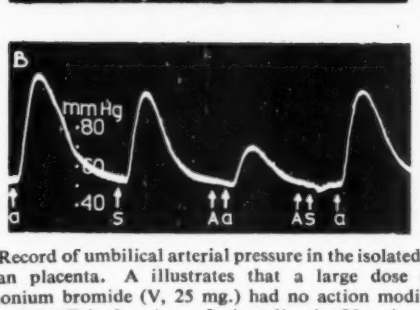

FIG. 4.—Record of umbilical arterial pressure in the isolated perfused human placenta. A illustrates that a large dose of hexamethonium bromide (V, 25 mg.) had no action modifying the effect of 5-HT (s, 2 μg.) or of adrenaline (a, 25 μg.). Note the biphasic response to 5-HT. B illustrates that the ganglion blocking agent trimetaphan (A, 10 mg.) in large doses completely abolished the action of 5-HT (s, 1 μg.), while the adrenaline effect (a, 10 μg.) was less affected.

Another ganglionic blocking agent, trimetaphan, was also tested. Small doses were ineffective, but larger doses (5 to 10 mg.) antagonized the action of subsequently added 5-HT. The effect of adrenaline was similarly, but to a lesser degree, affected by previous injection of trimetaphan (Fig. 4B).

Heparin.—The possible pharmacological action of heparin was studied in 6 preparations in doses ranging from 1 to 30 mg. The action of 5-HT was tested at intervals between injections of increasing doses of heparin. No modifying action on the 5-HT effect could be observed, nor did the general sensitivity of the preparation decrease after these doses.

Discussion

Some previous authors (Reid and Rand, 1952; Freyburger, Graham, Rapport, Seay, Govier, Swoap, and Vander Brook, 1952) have demonstrated that 5-HT shows tachyphylaxis in different preparations. Although tachyphylaxis could not usually be demonstrated in the placenta experiments reported here, it should be pointed out that large doses could not be used because they would cause a complete closure of the vessels.

Some of the antagonists investigated in this study belong to the group of adrenaline antagonists and have been shown to antagonize the action of 5-HT. This is exemplified by phentolamine, which blocks the action of both 5-HT and adrenaline. For both types of action, however, rather large doses were needed (1 to 3 mg.). Phentolamine has previously been found to antagonize the action of 5-HT in the perfused isolated guinea-pig lung (Bhattacharya, 1955). In the placenta preparation dihydroergotamine has also been shown to abolish the action of 5-HT as well as it does the action of adrenaline (Eliasson and Åström, 1955).

Chlorpromazine, which amongst other properties possesses anti-adrenaline effects, has been found to antagonize the action of 5-HT in the placenta. This action of chlorpromazine has previously been reported by Costa (1956) on uterine contractions, by Gyermek, Lázár, and Csák (1956) on the isolated rat uterus, and by Lecomte (1955) on the nictitating membrane of the cat. Potent 5-HT antagonism by chlorpromazine was also demonstrated by Benditt and Rowley (1956) in the rat *in vivo* and on the rat colon *in vitro*.

Yohimbine is a further example of a drug which shows both anti-adrenaline and anti-5-HT properties. As an anti-5-HT compound yohimbine is quite potent in the placenta preparation. The antagonism between yohimbine and 5-HT has been previously studied in other preparations (isolated segments of carotid arteries) by Shaw and Woolley (1953), who first recognized that yohimbine was a structural analogue of 5-HT.

A second group of anti-5-HT compounds are more specific in nature and do not exert any appreciable anti-adrenaline action. This is true especially for LSD, which in small doses completely abolishes the action of 5-HT. Tryptamine in small doses also abolishes the action of 5-HT without significantly altering the adrenaline response. LSD and tryptamine both have a direct vasoconstrictor action if administered in sufficient doses. A constrictor action of LSD on other isolated perfused vascular areas (for example, the rabbit ear) has earlier been demonstrated by Ginzel and Kottegoda (1953) and Savini (1956). The first-mentioned authors also reported that tryptamine was about 10 to 25 times less effective than 5-HT in causing vasoconstriction in the rabbit ear.

Mescaline in small doses caused a slight enhancement of the 5-HT action in some preparations, and larger doses always had a constrictor action on the umbilical vessels. An increase of the action of 5-HT on the isolated uterus after small doses of mescaline has previously been reported by Costa (1956), who also found that large doses of mescaline caused uterine contractions.

Of the ganglionic blocking agents tested, hexamethonium and tetraethylammonium were inactive in the placenta preparation and the actions of 5-HT and adrenaline remained the same even after high doses of these compounds. In the case of hexamethonium, a potentiation of the action of 5-HT was sometimes observed in the isolated guinea-pig ileum by Rocha e Silva, Valle, and Picarelli (1953). A local peripheral vasodilator action with tetraethylammonium chloride was found by Siems and Rottenstein (1950) in the hind limb of the dog. Augmented activity of different vasoactive substances after large doses of tetraethylammonium in dogs, cats, rats, and man has been demonstrated by Page and Taylor (1950).

In the group of ganglionic blocking agents tested, only trimetaphan was found to be effective in the placenta preparation. In large doses it antagonized the action of 5-HT and to a lesser degree that of adrenaline. Evidence that trimetaphan has direct peripheral actions, besides its ganglionic blocking properties, has earlier been presented by McCubbin and Page (1952).

Reserpine, which has been reported by Costa (1956) to antagonize 5-HT induced uterine contractions, was found to be ineffective in the

placenta preparation. The same was true also for heparin even in very high doses. Heparin was studied in this investigation, since it had previously been demonstrated that heparin exerts an antagonistic effect on the action of 5-HT *in vivo* and *in vitro* (Smith and Smith, 1955). An antagonism of this kind would be of great interest in clinical cases of embolism or thrombosis. A local release of 5-HT could, by way of vasoconstriction, contribute to the impairment of the local circulation in such patients (Comroe, van Lingen, Stroud, and Roncoroni, 1953).

The results obtained in this investigation on a non-innervated preparation speak in favour of a direct mode of action of 5-HT on the smooth muscles of the vessels. The antagonists included in this study exerted their action in this preparation in the same manner as has previously been demonstrated in different innervated organs. This indicates also that the mechanism of antagonism is independent of the presence of nervous structures.

The fact that 5-HT has strong vasoconstrictor actions in different local vascular regions, and the observation that the mechanism of antagonism also seems to be located at the smooth muscles of the vessels, are of particular interest for the study of psychotic effects of some of these compounds. Even if the results obtained in the placenta and other isolated vascular beds cannot directly be applied to the cerebral vessels it seems worth considering that some of the changes observed in psychic functions, particularly after local administration of the drugs into the ventricles of the brain, might be produced as a result of altered blood supply to different central nervous structures.

If the results obtained in this study are compared with those of Ginzel and Kottegoda (1953), who studied the effect of 5-HT and some of its antagonists on the lung circulation, great similarity is revealed. This illustrates further the point brought out by Euler (1938), that striking similarity seems to exist between the pharmacological responses of the umbilical and the lung vessels.

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References

Benditt, E. P., and Rowley, D. A. (1956). *Science*, **123**, 24.
Bhattacharya, B. K. (1955). *Arch. int. Pharmacodyn.*, **103**, 357.
Comroe, J. H., Jr., van Lingen, B., Stroud, R. C., and Roncoroni, A. (1953). *Amer. J. Physiol.*, **173**, 379.
Costa, E. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 39.
Eliasson, R., and Åström, A. (1955). *Acta Pharmacol. Toxicol.*, **11**, 254.
Erspamer, V. (1954). *Pharmacol. Rev.*, **6**, 425.
Euler, U. S. v. (1938). *J. Physiol.*, **93**, 129.
Freyburger, W. A., Graham, B. E., Rapport, M. M., Seay, P. H., Govier, W. M., Swoap, O. F., and Vander Brook, M. S. (1952). *J. Pharmacol.*, **105**, 80.
Ginzel, K. H., and Kottegoda, S. R. (1953). *Quart. J. exp. Physiol.*, **38**, 225.
Guarna, A. (1934). *Ann. Ostet. Ginec.*, **56**, 1231.
Gyermek, L., Lázár, I., and Csák, A. Zs. (1956). *Arch. int. Pharmacodyn.*, **107**, 62.
Lecomte, J. (1955). Ibid., **100**, 457.
McCubbin, J. W., and Page, I. H. (1952). *J. Pharmacol.*, **105**, 437.
Page, I. H., and Taylor, R. D. (1950). *Circulation, N.Y.*, **1**, 1233.
Reid, G., and Rand, M. (1952). *Nature, Lond.*, **169**, 801.
Rocha e Silva, M., Valle, J. R., and Picarelli, Z. P. (1953). *Brit. J. Pharmacol.*, **8**, 378.
Savini, E. C. (1956). Ibid., **11**, 313.
Schmitt, W. (1922). *Z. Biol.*, **75**, 19.
Shaw, E., and Woolley, D. W. (1953). *J. biol. Chem.*, **203**, 979.
Siems, L. L., and Rottenstein, H. S. (1950). *Amer. J. med. Sci.*, **220**, 649.
Smith, G., and Smith, A. N. (1955). *Surg. Gynec. Obstet.*, **101**, 691.

Brit. J. Pharmacol. (1957), **12**, 415.

THE ADRENALINE AND NORADRENALINE CONTENT OF THE ADRENAL GLAND OF THE CAT FOLLOWING DEPLETION BY ACETYLCHOLINE

BY

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This paper describes the attempts that were made to obtain a replacement of adrenaline and noradrenaline in the adrenal gland of the atropinized cat, subsequent to the depletion of the gland by repeated intravenous doses of acetylcholine. In the anaesthetized animal, there was no replacement of adrenal amines within 15 hours of the depletion. The further loss of amine that occurred during this time was prevented by the denervation of the gland. If the animal was allowed to recover from the anaesthetic there was some replacement of amines within 2 to 3 days, provided that the condition of the animal was satisfactory. By 6 to 7 days the total amine content had returned to its initial level, but there was now an alteration in the relative proportions of the two amines. Although the adrenaline was still well below the resting level, the noradrenaline was several times its initial value. By one month, the noradrenaline had decreased and the adrenaline had increased to their initial amounts and proportions. Thus this work gives evidence for the formation of adrenaline from noradrenaline.

Many workers have shown that various substances cause the release of catechol amines from the adrenal gland, but few have studied the subsequent replacement of these amines. One of the earliest papers in this field was by Crowden (1929), who depleted the adrenal glands of cats by exposing the animals to a low environmental temperature. He found that there was complete recovery of the adrenaline content in three days and that this occurred in the denervated gland to as great an extent as in the innervated gland. Van Arman (1950) showed that, three days after depletion of rat adrenal glands with physostigmine, the denervated gland contained as much amine as the innervated gland.

These workers only assayed the adrenal glands for total amine, calculated as adrenaline. The first mention of noradrenaline in this type of work was by Burn, Hutcheon, and Parker (1950), who studied insulin depletion in the adrenal glands of rats. Their evidence indicated that noradrenaline is a precursor of adrenaline and that, after depletion of the gland for several hours, the supply of noradrenaline is restored more rapidly than it can be methylated to form adrenaline. Hökfelt (1951) found that it took about six days for the catechol amine stores in the rat gland to be restored, following insulin depletion. More recently, Holland and Schümann (1956), studying the effect of excitation of the splanchnic nerve on the adrenal amines of the cat, suggested that splanchnic stimulation increases the rate of synthesis and methylation of noradrenaline.

Thus it may be seen that in these few papers the variety of species, of experimental conditions, and of depleting agents makes it difficult to reach any definite conclusions as to the rate and nature of the synthesis of the catechol amines in the depleted adrenal gland. Butterworth and Mann (1957b) have shown that acetylcholine releases similar percentages of adrenaline and noradrenaline from the adrenal gland of the cat, and they observed no replacement during the period of liberation of the amines. This paper reports the course of replacement of the adrenal amines following depletion of the adrenal glands by acetylcholine.

METHOD

Healthy, adult cats of both sexes were used.

Short-term Experiments

The animal was given 6 mg./kg. of atropine sulphate intraperitoneally and anaesthetized with ether and chloralose as described in the previous paper (Butterworth and Mann, 1957b). Both adrenal glands

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were depleted of their adrenaline and noradrenaline by repeated intravenous doses of acetylcholine chloride. The dose (0.2 to 3.0 mg./kg.) and the number of doses were such as to cause an approximately 50% depletion. This was estimated by a comparison of the acetylcholine-induced rises in blood pressure with pressor responses to doses of adrenaline and noradrenaline. Following the depletion, one gland was removed as a control depleted gland and the animal was left on a warm operating table for a number of hours. An attempt was made to remove the second gland, at varying time intervals, while the animal was in good condition (as judged by its blood pressure and respiration). In the latter experiments the splanchnic nerve fibres to the second adrenal gland were cut when the first gland was removed. Control experiments were performed in which no acetylcholine was given but one gland was denervated and the animal allowed to survive for a number of hours.

Long-term Experiments

The cat was given 6 mg./kg. of pentobarbitone sodium subcutaneously and 6 mg./kg. of atropine sulphate intraperitoneally. Ten minutes later ether was administered in an anaesthetic chamber, and open ether anaesthesia was maintained throughout. Injections were made into the femoral vein and the blood pressure was recorded from the femoral artery of the same limb. Two series of experiments were performed. In Series 1 both glands were depleted with acetylcholine, as in the short-term experiments, and then one gland was removed as a depleted control. In Series 2 one gland was removed as a non-depleted control and then the other depleted in the usual way. After depletion and unilateral adrenalectomy a solution of penicillin sodium was placed in the abdominal cavity, the abdominal walls were sutured together, the femoral artery and vein ligatured and the cut edges of the skin held together with Michel's clips. Aseptic precautions were taken throughout and penicillin was given subcutaneously at the end of the operation. The animal was carefully looked after during the post-operative recovery period. When the second gland was due to be removed (see later) the animal was re-anaesthetized and the adrenal gland removed as in the first part of the experiment.

The results of Series 1 were intended to show the extent to which the amines had been replaced in comparison with the depleted gland, and those of Series 2 whether the amine content had returned to its initial, non-depleted level. In both series the second gland was removed at 2 to 3 days, 6 to 7 days or one month after the first adrenalectomy. Separate control experiments were performed at these various time intervals.

In all experiments, the removal and extraction of the adrenal glands and the subsequent biological and chromatographic analysis of the extracts were made using the methods described by Butterworth and Mann (1957b). The adrenaline and noradrenaline contents of the adrenal glands are expressed as μg./gland (Butterworth and Mann, 1957a) in terms of the laevo isomers of the base.

Results

It has been shown in a previous paper (Butterworth and Mann, 1957b) that acetylcholine causes a similar % depletion of adrenaline and of noradrenaline. Hence in the following experiments any difference in the degree of depletion of the amines must be due to some other factor.

Short-term Experiments

In the experiments in which the innervation of the adrenal gland was intact (Table I) there was, in all but two animals, a further loss of both amines. The longer the time interval between the removal of the two glands, the greater was the loss. If the animal died suddenly after maintaining its initial blood pressure for a number of

TABLE I

SHORT-TERM EXPERIMENTS

Both adrenal glands were depleted of their amines by acetylcholine. One gland was then removed and the other remained in the cat for the number of hours given in the column headed "Interval." The amine content of the second adrenal gland is compared with the first.

Cat No.	Interval (hr.)	Condition of Animal	Total Amine Difference in μg.	% Adrenaline Difference	% Noradrenaline Difference
Group A—Similar % loss of adrenaline and noradrenaline.					
1	0·5	Died suddenly	−11·8	−11·1	−1·6
2	3·5		−40·9	−12·1	−11·4
3	3·5		−138·1	−38·1	−28·2
4	7·0		−179·9	−66·5	−65·5
5	7·0		−120·0	−66·8	−77·2
6	17·0		−265·2	−74·7	−88·3
		Mean		−44·9	−45·4
Group B—Greater % loss of noradrenaline.					
7	3·0	Gradual fall in blood pressure	−53·4	−9·5	−53·6
8	4·0		−102·5	−15·4	−50·3
9	4·0		−190·9	−18·4	−75·2
10	4·0		−59·6	−7·3	−29·8
11	4·5		−30·7	−5·1	−16·3
12	5·0		−72·5	−1·5	−48·9
13	5·0		−146·5	−20·1	−77·2
14	5·25		−198·9	−32·7	−90·3
15	6·0		−175·1	−32·2	−58·6
16	9·0		−104·3	0	−70·4
		Mean		−14·2	−57·1
Group C—Greater % loss of adrenaline.					
17	3·0	Good	+3·6	−30·7	+19·6
18	6·5		−32·1	−37·2	+57·2
19	15·25		−48·1	−47·0	−21·3
		Mean		−38·3	+18·5
In the following groups the gland which remained was denervated immediately after both glands had been depleted.					
Group D—Little alteration in adrenaline or noradrenaline.					
20	1·0	Poor	+3·6	−2·9	+7·6
21	4·75		−6·5	+10·8	−10·4
22	6·5		−28·1	−11·0	−10·7
23	7·75		+3·7	+2·1	+1·8
24	9·0		+3·3	−12·5	+21·1
25	13·0		−15·7	−19·2	+7·3
		Mean		−5·5	+2·8
Group E—Some replacement of amines.					
26	5·0	Good	+19·3	+25·9	+1·4
27	7·75		+30·7	+28·5	+10·9

hours (Table I, Group A), there was a similar % loss of adrenaline and of noradrenaline. Probably this was due to the basal liberation from the gland since, when this loss was calculated in μg./kg./min., it was comparable to the value obtained in the control series, where the amine contents of the innervated and denervated glands were compared. If the blood pressure fell gradually and the second gland was not removed until the pressure was about 40 to 50 mm. Hg (Group B) there was a greater % loss of noradrenaline than of adrenaline. This may have been caused by the gradual fall in the blood pressure of these animals. Under such circumstances it might be expected that there would be a preferential liberation of the more potent vasopressor amine, namely noradrenaline. If the condition of the animal was good (Group C), although there was a loss of adrenaline, there was some evidence of a little replacement of noradrenaline. This is similar to some of the results in the long-term experiments.

To prevent this reflex liberation of amines during the recovery period, the splanchnic nerve fibres to the second gland were cut when the first gland was removed. (Control experiments showed that while the innervated gland lost amine at about 0.07 μg./kg./min., the liberation from the denervated gland was prevented.) In those animals which were in poor condition (Table I, Group D), there was little change in either the adrenaline or the noradrenaline content of the gland for a period of up to 13 hr. The glands of the two cats that were in good condition (Group E) showed a small increase in amine content. Thus it was obvious that, although denervation prevented the loss of amine from the gland, the period of time for which the animal could survive in good condition under anaesthesia was insufficiently long to allow any appreciable increase in amine content to occur. Therefore it was necessary to perform long-term experiments.

Long-term Experiments

Series 1.—In this series the control glands were depleted of their amine content. Thus in these results a negative % difference of amine in the second gland indicated that the gland had lost amine during the recovery period, while a positive sign indicated a replacement of amine. Cat Nos. 28, 29, and 30 (Table II) were in poor condition when the second gland was removed two days after depletion, and there was a further loss of both adrenaline and noradrenaline from these glands. Cat Nos. 31 to 35 were in fair or very good condition. Of these, the glands of cat Nos. 31 and 33 showed a further overall loss of amine.

TABLE II

LONG-TERM EXPERIMENTS

In Series 1 both adrenal glands were depleted of their amines by acetylcholine before removal of the first gland. In Series 2 one adrenal gland was removed before depletion of the second gland. In each series the number of days between the removal of the glands is given in the column headed "Interval." The amine content of the second adrenal gland is compared with the first.

Cat No.	Interval (Days)	Post-operative Condition of Animal	Total Amine Difference in μg.	% Adrenaline Difference	% Noradrenaline Difference
Series 1					
28	2	Poor	−262·5	−95·2	−73·8
29	2		−115·3	−44·0	−57·9
30	2		−98·3	−45·0	−51·3
31	2	Fair	−44·2	−97·3	+35·5
32	2		−0·1	−66·4	+34·2
33	2	Very good	−52·5	−33·4	+18·6
34	3		+41·1	+4·5	+58·2
35	3		+14·2	+9·9	+162·8
36	7	Poor	147·4	−77·8	−44·4
37	7		−31·6	−46·4	−42·5
38	7	Fair	+13·3	−39·0	+34·8
39	7	Very good	+79·0	+62·9	+102·9
40	6		+104·5	+20·5	+295·3
41	6		+150·3	+21·6	+319·6
42	7		+99·7	+11·7	+481·8
43	27	Poor	+7·2	+10·3	0
44	28	Good	+106·9	+60·0	+47·1
45	27		+162·6	+89·5	+84·5
46	28		+187·5	+101·7	+92·9
47	28	Very good	+254·1	+122·6	+104·9
48	29		+165·1	+199·9	+192·6
Series 2					
49	2	Poor	−113·8	−76·6	−52·9
50	2	Good	−171·6	−46·5	−33·8
51	7	Fair	−91·4	−56·4	−11·1
52	7	Good	−116·9	−49·0	−30·3
53	7		+41·2	−38·9	+48·6
54	6		−18·1	−56·3	+378·8
55	30	Good	+18·9	+8·3	−0·6
56	29		+13·0	+3·8	+9·2
57	30	Very good	+50·4	+18·3	+16·7
58	29		+80·3	+26·8	+23·7

those of cat Nos. 32 and 35 just maintained their total amine content, and that of cat No. 34 showed an increase in total amine. However, in all five there was an increase in the noradrenaline content.

Of the animals in which the second gland was removed after six to seven days (Table II), cat Nos. 36 and 37 were in poor condition and their glands lost further amine. Cat No. 38 was in fair condition and its adrenal gland just maintained its total amine content. Cat Nos. 39 to 42 were in very good condition and their glands showed a marked increase in total amine content. In all four there was a considerably greater % increase in noradrenaline than in adrenaline. For example, in cat No. 42 there was a 481.8% increase in the noradrenaline content of the gland but only an 11.7% increase in the adrenaline content.

Considering those animals in which the second gland was removed one month after depletion (Table II), only cat No. 43 was in a poor post-

operative condition and it just maintained its adrenal amine content. All the others (cat Nos. 44 to 48) were in good or very good condition, and the glands showed a considerable increase in total amine. However, in contrast to the results of the six to seven day experiments, the % increases of adrenaline and noradrenaline were similar.

Series 2.—Here the control gland was not depleted. In these experiments the exact degree of depletion of the experimental gland was not known, but a 50% loss may be presumed. Thus it was inferred that a negative % difference of less than 50 indicated a replacement of amine, the smaller this negative % the greater the increase in amine content. A positive % difference showed an increase in amine such that the second gland contained more than it did originally. It may be seen (Table II) that in the glands of cat Nos. 49 and 50 two days after depletion there was a slightly smaller % depletion of noradrenaline than of adrenaline. The glands of cat Nos. 51 and 52 examined after seven days showed some increase in noradrenaline but not in adrenaline, while those of cat Nos. 53 and 54 showed a marked increase in noradrenaline but again little change in adrenaline. By one month, the adrenaline and noradrenaline contents of the adrenal glands of cat Nos. 55 to 58 had returned to the original non-depleted values. Actually with cat Nos. 57 and 58 the amine content of the gland that was originally depleted was a little higher than that of the control gland. This may be ascribed to the fact that these two animals were in a much better condition at the end of the experiment than they were at the beginning.

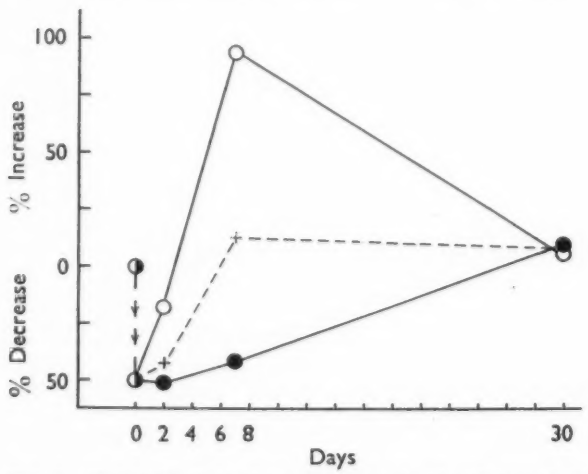


FIG. 1.—Composite diagram to show the rate of replacement of adrenaline (●), of noradrenaline (○) and of total amines (+) in the adrenal gland of the cat at the various time intervals following depletion. ◑ denotes adrenaline and noradrenaline. All results are expressed as % of the initial, non-depleted level.

The mean of the results from animals in good post-operative condition in Series 1 and 2 has been plotted in Fig. 1. It may be seen that, whereas the adrenaline content slowly increased to its non-depleted level by one month, the noradrenaline content exceeded its original value in seven days and then decreased to its normal amount by one month. Although it took one month for the normal proportions of adrenaline and noradrenaline to be regained, the total amine content had returned to normal by seven days and subsequently was maintained at this level.

Control Experiments

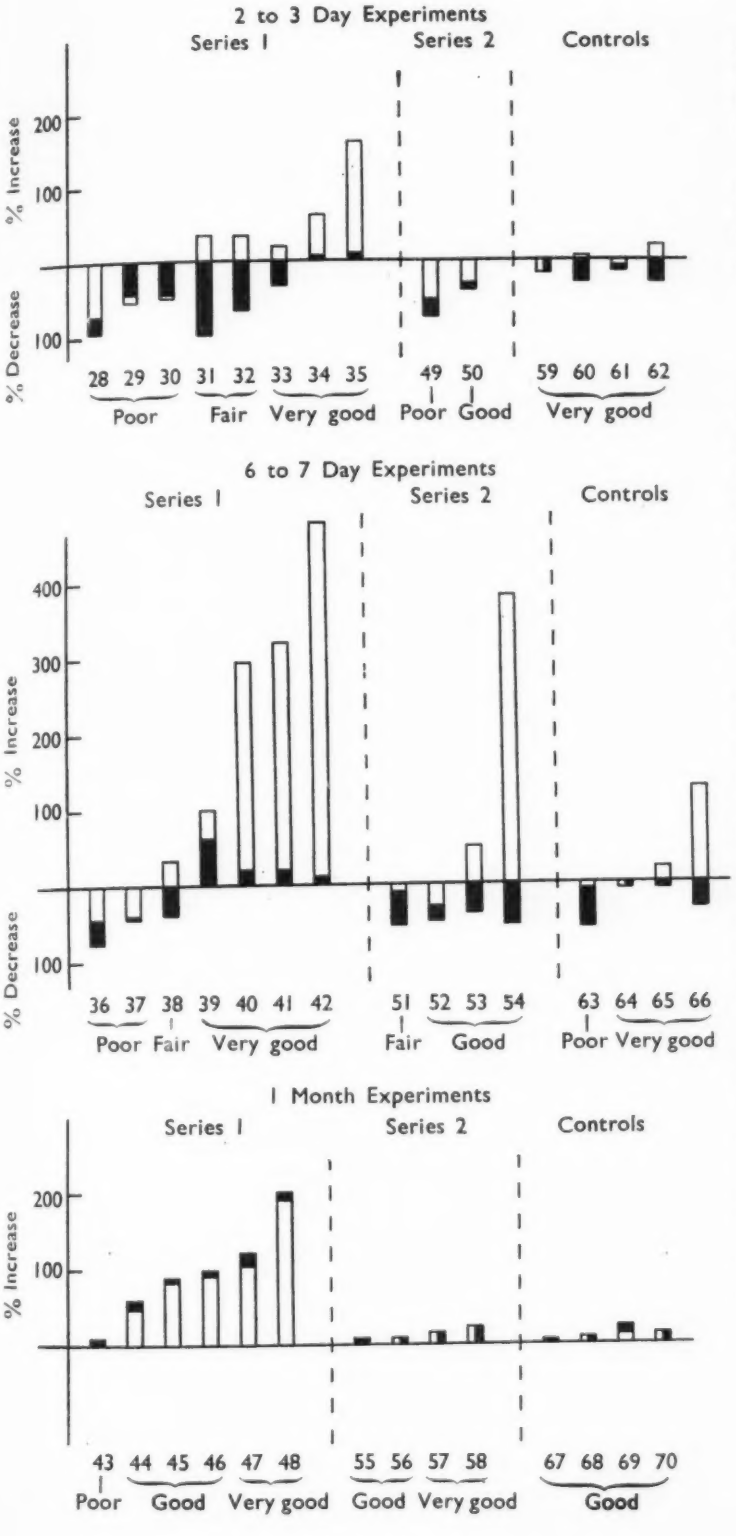
In addition to using one gland of each cat as a control, further control experiments were performed. In each of these experiments the adrenal gland of one side was removed and its amine content measured. Then after an interval of two to three days, six to seven days, or one month, the remaining gland was removed for assay. In all but one experiment out of 12 there was little difference between the total amine contents of the two glands (Table III). Nevertheless the small changes which occurred followed a similar trend to those in Table II. With the two to three day and the six to seven day experiments, the % noradrenaline difference between the two glands was either similar to or a smaller negative or a larger positive quantity than the % adrenaline difference. In the experiments lasting one month the % noradrenaline difference was very similar to that of the adrenaline.

TABLE III

CONTROL LONG-TERM EXPERIMENTS

In these experiments no acetylcholine was given. The number of days between the removal of the two glands is given in the column headed "Interval."

Cat No.	Interval (Days)	Post-operative Condition of Animal	Total Amine Difference in μg.	% Adrenaline Difference	% Noradrenaline Difference
59	2	Very good	−34·0	−17·5	−17·4
60	2		−17·1	−29·6	+2·6
61	3		−18·6	−12·7	−6·9
62	2		−37·2	−31·8	+21·2
63	7	Poor	−94·6	−61·8	−8·0
64	7	Very good	−17·1	−5·1	−9·0
65	6		+0·5	−9·8	+21·7
66	7		−11·4	−36·7	+124·9
67	28	Good	+3·3	+4·5	−1·0
68	28		+16·4	+7·4	+6·9
69	31		+41·8	+26·3	+12·5
70	34		+36·8	+16·7	+13·3



A comparison of the results of Series 1 and Series 2 with those of the control experiments at two to three days, six to seven days, and one month is shown in Fig. 2.

By means of the chromatographic analysis the presence of adrenaline and noradrenaline in all the glands was confirmed. No other catechol amines were detected.

Discussion

The results of the short-term experiments showed the necessity for experiments of longer duration. In the latter experiments a comparison was made between the amine content of the control gland and that of the second gland, removed some days after the first. An increase in the amine content of the second gland, expressed as a % of that of the first, was termed the degree of replacement. This replacement of amines must be somewhat greater than the amount measured because, at the same time as the gland was re-forming amine, it was also liberating a small basal quantity. The assays measured only the store of amines in the gland at a given time. In order to minimize this basal liberation of amines, denervation of the gland would be necessary, but this, in its turn, might affect the process of synthesis.

The results of Series 1 recorded such replacement of adrenaline and noradrenaline as had taken place. The results of Series 2 enabled an estimate to be made of the time required for complete replacement of the amines. Thus the results of both series, taken together, give

Fig. 2.—The % difference of adrenaline (■) and noradrenaline (□) of the second adrenal gland of the cat, as compared with the first (control), at various time intervals after removal of the first gland. In Series 1 the control gland was depleted, and hence any replacement of amines is shown by the columns above the abscissa. In Series 2 the control gland was not depleted, and any value above the 50% depletion level indicates replacement of amine. The numerals refer to the cat Nos. in the tables. The post-operative condition of each cat is recorded below the cat Nos.

some indication as to the rate at which the amines are replaced in the gland. Although the results of each experiment varied, there was a similar trend throughout. Considering only those animals that were in good post-operative condition, there was some replacement of noradrenaline at two to three days, and the quantity of this amine present at six to seven days was considerably more than initially. In contrast to this, there was no increase of adrenaline at two to three days and only a small increase by seven days. By one month noradrenaline had decreased and adrenaline had increased to their initial amounts and proportions. However, the total amine content had returned to normal by seven days, and thereafter it was maintained at this level. This relatively slow rate of formation of the amines is in agreement with the work of Hökfelt (1951) and others, who found a slow increase in the amine content of the gland following insulin depletion, but is in contrast to the work of Holland and Schümann (1956). They obtained some evidence of synthesis while the gland was being depleted by splanchnic nerve stimulation, and thus they suggested that this more rapid rate of synthesis might be due to the electrical stimulation of the nerve. In this connexion, Bülbring (1949) has shown that adrenal glands removed after prolonged splanchnic stimulation possess a higher methylating capacity than normal glands. It is possible for the rates of formation to vary according to the experimental conditions and the type of depleting agent.

Although the change in amine content was very small in the control long-term experiments, the same shift in the balance of amines was observed as in both series of depletion experiments. This indicates that the greater degree of replacement of noradrenaline is not solely a result of the depletion of the gland by the doses of acetylcholine, but may occur physiologically. The loss of a small amount of adrenaline and noradrenaline was probably due partly to the anaesthetization of the animal and partly to the excitement that occurred during the recovery from the anaesthetic.

The most important factor affecting the amine content of the gland, subsequent to the depletion with acetylcholine, appears to be the condition of the animal. When the post-operative recovery of the cat was good the amines were replaced, the degree of replacement depending upon the time interval between the removal of the two glands. When, on the other hand, the animal was in poor post-operative condition, the second gland lost a further quantity of amine.

It has long been established (Elliott, 1912) that general anaesthesia causes a certain release of catechol amines from the adrenal gland. Whatever depletion occurred due to the ether in the first part of the experiment, also occurred when the animal was re-anaesthetized. Thus both glands of the animal were affected. A greater factor in this connexion is the excitement which may occur during the immediate post-operative recovery phase. This probably accounts for the small depletion of the second gland in the control experiments lasting two days.

No study of the adenosine triphosphate (ATP) content of the adrenal glands has been made, but it is of interest that Carlsson and Hillarp (1956) have found that morphine given to cats causes a similar % depletion of catechol amines and of ATP from the glands. It might be useful to investigate whether the increase in ATP content of the glands following depletion is similar to the increase in adrenaline and noradrenaline. Giving ATP during the recovery phase might hasten the formation of the catechol amines. The administration of a methyl donor substance, such as was used by Bülbring and Burn (1949), during the recovery phase might also be of interest.

Hillarp and Hökfelt (1953), Eränkö (1955) and others have studied the histology of the adrenal glands following depletion. Hillarp and Hökfelt have suggested that " All the cells of the adrenal medulla can form noradrenaline while only certain specific cells are able to effect a mechanism for the methylation of noradrenaline to adrenaline and to store this hormone. Those cells which lack this methylation capacity store noradrenaline." It would be of interest to study the histology of these cat adrenal glands at seven days and at one month after depletion. It might be expected that at seven days most of the medullary cells contain noradrenaline, while at one month some of these cells now contain adrenaline. Experiments of the type described above are now in progress.

The aim of the present study was to obtain some evidence of the rate of synthesis of adrenaline and noradrenaline. It has been shown (Butterworth and Mann, 1957b) that acetylcholine causes a similar % loss of both amines. The results of the long-term experiments suggest that there is an initial replacement of noradrenaline and a subsequent conversion of the noradrenaline to adrenaline ; the rate of methylation thus being slower than the rate of noradrenaline formation. Other possible explanations of these results are that either there was an

independent synthesis of the two amines, or there was a preferential liberation of one or other amine occurring concurrently with the synthesis, or that more than one mechanism was working at any one time. However, it is improbable that there was an independent synthesis because, if that were so, it is unlikely that noradrenaline would increase to an amount greater than its original value and then subsequently return to its normal value. It is very likely that there is a basal liberation occurring concurrently with the replacement of amines, but it is probable that this would be of similar percentages of the two amines, as was found in some of the experiments. The possibility of more than one mechanism occurring at any one time cannot be ignored, for example the depleted gland could take up adrenaline and noradrenaline from the blood as well as synthesizing these amines. However, van Arman (1951) has shown that the rat adrenal gland does not take up either amine from the blood stream. Thus this work gives strong support for the view that adrenaline is formed from noradrenaline in the adrenal gland of the cat.

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References

van Arman, C. G. (1950). *Amer. J. Physiol.*, **162**, 411.
—— (1951). Ibid., **164**, 476.
Bülbring, E. (1949). *Brit. J. Pharmacol.*, **4**, 234.
—— and Burn, J. H. (1949). Ibid., **4**, 202.
Burn, J. H., Hutcheon, D. E., and Parker, R. H. O. (1950). Ibid., **5**, 417.
Butterworth, K. R., and Mann, M. (1957a). *J. Physiol.*, **136**, 294.
—— —— (1957b). *Brit. J. Pharmacol.*, **12**, 422.
Carlsson, A., and Hillarp, N.-Å. (1956). *Acta physiol. scand.*, **37**, 235.
Crowden, G. P. (1929). *J. Physiol.*, **68**, 313.
Elliott, T. R. (1912). Ibid., **44**, 374.
Eränkö, O. (1955). *Ann. Med. exp. Fenn.*, **33**, 278.
Hillarp, N.-Å., and Hökfelt, B. (1953). *Acta physiol. scand.*, **30**, 55.
Hökfelt, B. (1951). Ibid., **25**, Suppl. 92.
Holland, W. C., and Schümann, H. J. (1956). *Brit. J. Pharmacol.*, **11**, 449.

Brit. J. Pharmacol. (1957), **12**, 422.

THE RELEASE OF ADRENALINE AND NORADRENALINE FROM THE ADRENAL GLAND OF THE CAT BY ACETYLCHOLINE

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The adrenal glands of atropinized cats were depleted of their content of adrenaline and noradrenaline by repeated intravenous doses of acetylcholine. In each experiment there was a similar percentage loss of both amines. This was irrespective of the degree of depletion, which ranged from 7.1% to 86.5%. In some experiments, adrenal blood was collected and it was found that the percentage of noradrenaline in the adrenal venous effluent (mean 62.0%) was similar to that in the amine lost from the depleted gland (mean 63.7%). In some experiments, the adrenaline and noradrenaline lost from the gland could be recovered quantitatively in the adrenal blood. The results indicate that, under these experimental conditions, synthesis is not taking place concurrently with the liberation of amines or that it is taking place so slowly as not to be detected.

One of the first workers to study the liberation of catechol amines from the adrenal gland was Elliott (1912). He studied the effects of both chemical stimuli (for example, morphine, tetrahydro-β-naphthylamine, gaseous anaesthetics) and physical stimuli (such as splanchnic nerve stimulation, pressure on the glands) on the loss of adrenaline from the adrenal glands of cats. He found that the chemical means was considerably more effective in causing a depletion than the physical means. Elliott and other early workers in this field only assayed the adrenal glands for adrenaline, and many years elapsed before it was shown that the adrenal medulla of most species contain two catechol amines, adrenaline and noradrenaline. Euler (1950) was the first to suggest that these two hormones can be liberated independently, and support of this hypothesis has come from many workers. Burn, Hutcheon, and Parker (1950) and Hökfelt (1951) have shown that there is a greater loss of adrenaline than of noradrenaline from the adrenal gland of the rat, following the administration of insulin. Outschoorn (1952), using the same species, found that insulin, morphine, and tetrahydro-β-naphthylamine caused a significant loss of adrenaline but not of noradrenaline. In the cat he found that stimulation of the glands by acetylcholine, potassium chloride, or electrical stimulation of the splanchnic nerve caused an increase in the adrenaline and noradrenaline content of the adrenal venous effluent. In the acetylcholine experiments the adrenal glands were not assayed, but with splanchnic stimulation there was a similar % depletion of both amines. Dunér (1954) found a marked increase in the secretion of adrenaline into the venous effluent from the adrenal gland of the cat in response to insulin hypoglycaemia, while there was only a slight increase in the noradrenaline output.

Workers such as Brücke, Kaindl, and Mayer (1952), Euler and Folkow (1953), and Redgate and Gellhorn (1953) have studied the effect of electrical stimulation of the hypothalamus on the release of catechol amines from the adrenal gland. They found in the cat that the proportion of the two amines in the adrenal venous effluent varied considerably according to the area that was stimulated. Thus it was concluded that some of the adrenal medullary cells produce adrenaline and others noradrenaline, and that these are innervated by separate fibres with a different hypothalamic representation.

Since adrenaline and noradrenaline can be liberated from the adrenal gland independently, it was thought of interest to study the effect of the natural mediator of splanchnic nerve transmission, namely acetylcholine, on the liberation

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of the adrenal medullary amines in the cat. The degree of depletion of the glands, the relative amounts of adrenaline and noradrenaline lost from the depleted gland, the proportion of adrenaline to noradrenaline in the adrenal venous effluent, and the correlation between the amines lost from the gland and those found in the plasma have been studied.

METHOD

Healthy, adult cats of both sexes were used.

Series 1. Depletion Experiments.—The animal was given 6 mg. of atropine sulphate/kg. of body weight intraperitoneally and anaesthetized 10 min. later, in an anaesthetic chamber, with ether followed by 60 mg./kg. of chloralose intravenously. One adrenal gland was removed and an extract prepared (see below). The other gland was depleted of its catechol amines by repeated intravenous doses of acetylcholine. The dose and the number of doses varied in each experiment so that differing degrees of depletion could be obtained. The dose range was 0.2 to 3.0 mg./kg. of body weight. The approximate amount of amine liberated was determined by comparing the acetylcholine-induced pressor responses with the rises in blood pressure produced by doses of adrenaline and of noradrenaline. Following the last dose of acetylcholine the second gland was removed and an extract prepared as for the first gland.

Series 2. Depletion Experiments with Collection of Adrenal Venous Effluent.—The animal was prepared as in Series 1 and the right adrenal gland was removed as a control. The left lumbar vein was then cannulated towards the adrenal gland, after tying off the surrounding blood vessels, care being taken to leave the arterial supply to the gland intact. Heparin (1,000 units/kg.) was given intravenously. When the adrenal vein was temporarily occluded, blood flowed into the cannulated lumbar vein. Immediately after each dose of acetylcholine had been given, blood was collected into ice-cooled centrifuge tubes. Collection was for a little longer than the duration of the rise in blood pressure, as determined by giving acetylcholine without occlusion of the adrenal vein. Half of each blood sample was retained (see below). The other half of each sample was returned to the cat to ensure that the acetylcholine was exerting a pressor effect and also to minimize the loss of blood from the animal. In addition, suitable control blood samples were collected. In each experiment about 30 doses of acetylcholine were given. The dose range was similar to that of Series 1, although here the main factor was that the dose should be sufficient to cause a high concentration of amines in the adrenal venous effluent. Following depletion, the right adrenal gland was removed.

Preparation of the Extracts of the Adrenal Glands.—Immediately the adrenal gland was removed, it was freed of connective tissue, weighed on a microtorsion balance and a 50 mg./ml. extract prepared by grinding the gland in 0.1 N hydrochloric acid with 300 mg. of acid-washed sand/100 mg. of gland. After centrifugation at 5,000 rev./min. for 2 min., the supernatant fluid was withdrawn and stored in an airtight container at 4°.

Preparation of the Plasma Samples.—Each half sample of blood was centrifuged at 5,000 rev./min. for 3 min. immediately after collection. The plasma was withdrawn into a graduated measuring cylinder containing 2 to 3 drops of concentrated hydrochloric acid. The remaining cells were re-centrifuged and any further plasma obtained was added to the first sample. All the samples from one experiment were pooled, measured, and then stored at 4°.

Assay of the Adrenal Gland Extracts and the Plasma Samples.—The adrenaline and noradrenaline contents of the adrenal gland extracts and the plasma samples were determined by differential biological assays. The assay was first performed on the acutely denervated nictitating membrane of the cat. The animal was then given hexamethonium bromide and the samples were assayed on the blood pressure. At least two assays of each extract or plasma sample were performed on each preparation. The adrenaline and noradrenaline contents were calculated, as the laevo isomers of the bases, by the formula of Bülbring (1949). The amine contents of the adrenal glands are expressed in μg./gland in view of the findings of Butterworth and Mann (1957a). In calculating the adrenaline and noradrenaline content of the adrenal venous effluent it was necessary to determine the distribution of amines between the cell and the plasma fractions. In each experiment a correction factor was applied for the small amount (approximately 10%) of adrenaline and noradrenaline which was present in the cell fraction. In addition to the biological assays, a paper chromatographic analysis was made by the method of Shepherd and West (1951), which employs *n*-butanol-acetic acid-water as the solvent and potassium iodate to locate the catechol amines.

RESULTS

Series 1. Depletion Experiments.—A marked rise in blood pressure was obtained in response to each dose of acetylcholine. Initially repeated doses caused rises in blood pressure of a similar magnitude (Fig. 1), but gradually, as the gland became depleted, the responses became smaller. If the dose of acetylcholine was then increased, the response returned to its initial magnitude. In some experiments the doses of acetylcholine were increased both in size and in number until no further pressor response was elicited (Fig. 2). Comparing the amine contents of the depleted and the control glands, it was found that there was a similar % depletion of adrenaline and noradrenaline in every experiment (Fig. 3). This similar degree of depletion of both amines was independent of the degree of depletion of the

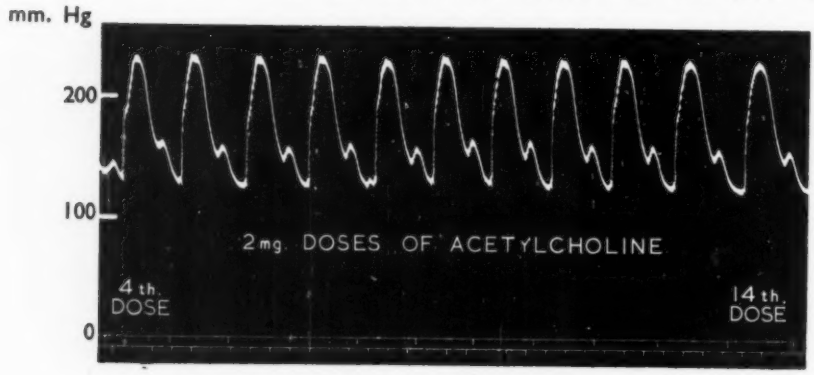

FIG. 1.—Cat (3.4 kg.), 6 mg./kg. of atropine sulphate intraperitoneally followed by ether and 60 mg./kg. of chloralose intravenously. The right adrenal gland was removed. The blood pressure tracing shows the similarity of successive responses to 2 mg. doses of acetylcholine given intravenously. Time, 30 sec.

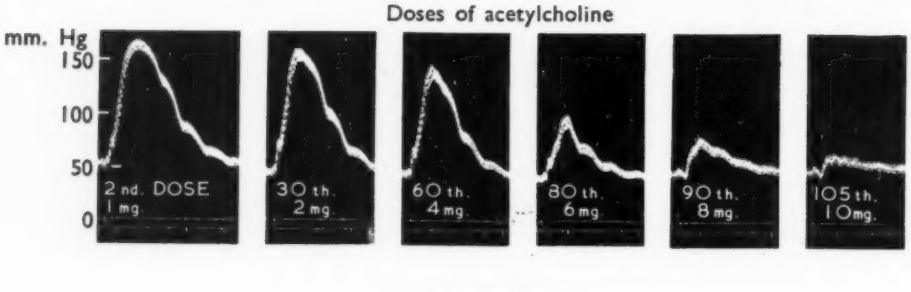

FIG. 2.—Cat (3.9 kg.), 6 mg./kg. of atropine sulphate intraperitoneally followed by ether and 60 mg./kg. of chloralose intravenously. The left adrenal gland was removed. The blood pressure tracing shows the ultimate diminution in the response to repeated intravenous doses of acetylcholine. Time, 30 sec.

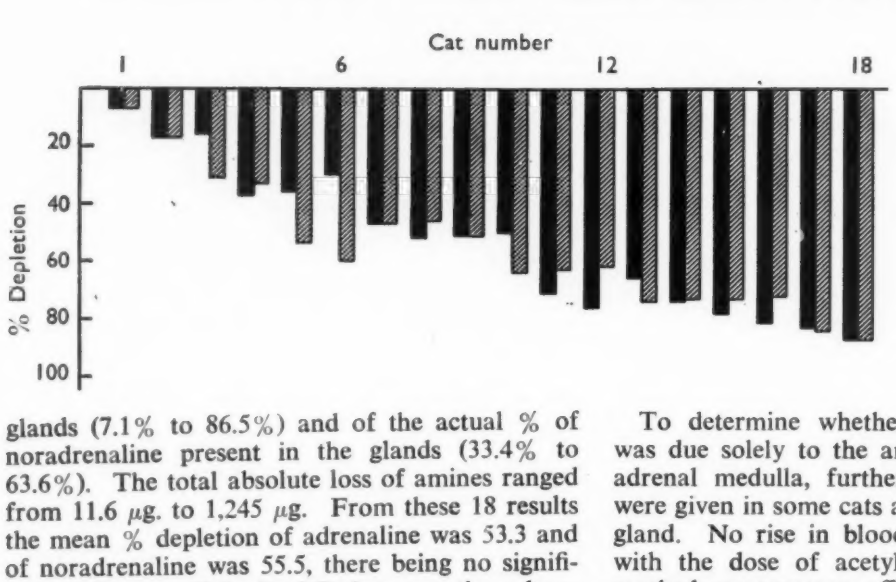

FIG. 3—Results of Series 1, showing the similarity in the percentage loss of adrenaline (■) and of noradrenaline (▨) when the adrenal gland of the atropinized cat is depleted by repeated doses of acetylcholine.

glands (7.1% to 86.5%) and of the actual % of noradrenaline present in the glands (33.4% to 63.6%). The total absolute loss of amines ranged from 11.6 μg. to 1,245 μg. From these 18 results the mean % depletion of adrenaline was 53.3 and of noradrenaline was 55.5, there being no significant difference ($0.8>P>0.7$) between the adrenaline depletion values and those of the noradrenaline.

In three cats a constant infusion of acetylcholine was given in an attempt to produce a continuous liberation of amine from the adrenal medulla and hence a greater depletion of the glands in a shorter period of time. At first a maintained rise in blood pressure was produced, but a marked tachyphylaxis soon developed and the blood pressure returned to normal while the acetylcholine infusion was still being given.

To determine whether the vasopressor effect was due solely to the amines liberated from the adrenal medulla, further doses of acetylcholine were given in some cats after removing the second gland. No rise in blood pressure was produced with the dose of acetylcholine which caused a marked vasopressor effect before the bilateral adrenalectomy, but in a few cases, on increasing the dose 10 to 20 times, a very small rise in blood pressure was produced.

Series 2. Depletion Experiments with Collection of Adrenal Venous Effluent.—As in Series 1, there was always a similar % depletion of adrenaline (mean 57.5%) and of noradrenaline (mean 56.3%) in each cat (Fig. 4). Again this similar % depletion of the two amines in each experiment was irrespective of the degree of depletion of the glands (35.3% to 78.5%) and of the actual

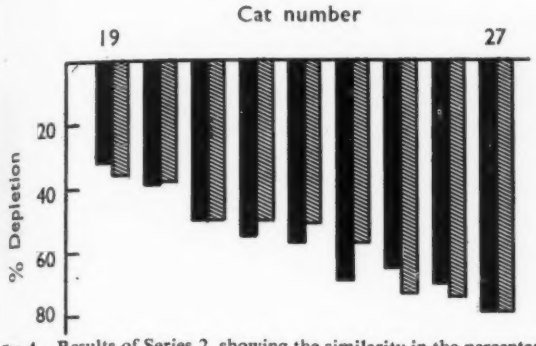

FIG. 4.—Results of Series 2, showing the similarity in the percentage loss of adrenaline (■) and of noradrenaline (▩) when the adrenal gland of the atropinized cat is depleted by repeated doses of acetylcholine.

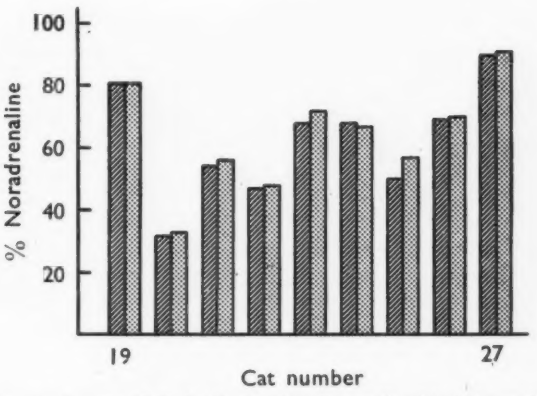

FIG. 5.—Results of Series 2, showing the relationship between the percentage of noradrenaline in the adrenal venous effluent (▩) and in the loss from the depleted adrenal gland (▨).

% of noradrenaline present in the glands (34.5% to 90.7%). There was no significant difference ($0.9>P>0.8$) between the depletion of adrenaline and of noradrenaline. In every experiment there was a similar % of noradrenaline in the control adrenal gland, the depleted gland and the plasma obtained from the adrenal venous effluent (Table I) and therefore between the % of noradrenaline

TABLE I

SERIES 2. DEPLETION OF CAT ADRENAL GLANDS BY ACETYLCHOLINE, WITH COLLECTION OF ADRENAL VENOUS EFFLUENT

Cat No.	% of Total Amines Lost from Depleted Gland	Total Amines Lost from Depleted Gland (μg.)	Total Amines in Adrenal Effluent (μg.)	% of Noradrenaline			
				Control Gland	Depleted Gland	Adrenal Effluent	Loss from Depleted Gland
19	35·3	149·9	151·7	79·1	78·0	81·0	81·0
20	38·5	144·8	104·0	34·5	35·5	31·6	32·9
21	50·0	242·1	222·4	55·8	55·7	53·9	55·9
22	52·2	128·9	124·3	50·6	53·1	46·8	47·6
23	53·0	222·5	108·9	73·6	76·1	68·3	71·4
24	60·4	170·4	144·9	71·0	77·4	67·6	66·8
25	69·4	208·9	214·3	54·5	48·4	50·0	57·3
26	73·1	164·2	78·0	68·3	65·1	68·6	69·5
27	78·5	778·5	420·5	90·7	90·8	90·2	90·7
			Mean=	64·2	64·5	62·0	63·7

in the adrenal venous effluent and in the loss from the gland (Fig. 5). The total amount of amines in the plasma samples was equal to or less than the total amount of amines lost from the depleted gland (Table I). In three experiments (Table I, cat Nos. 23, 26, and 27), the total amine content of the adrenal venous effluent was only approximately 50% of the total amine lost from the gland. This poor recovery is ascribed to the fact that these were the first three experiments in which adrenal blood was collected and there was a "leak" into the general circulation, due to incomplete ligation of the small blood vessels surrounding the gland. This "leak" was detected by the rise in blood pressure that occurred when each dose of acetylcholine was given. In those experiments where there was no such "leak" the recovery of amines was satisfactory. In no case did the control plasma samples possess any sympathomimetic activity.

By means of the chromatographic analysis the presence of adrenaline and noradrenaline in all the adrenal glands was confirmed. No other catechol amines were detected.

DISCUSSION

Acetylcholine always caused a similar % loss of adrenaline and noradrenaline, irrespective of whether the degree of depletion was small or large. This absence of preferential loss of either amine may be of some significance in connexion with the normal physiological functioning of the gland. A maximum depletion to 13.5% of the initial amount was obtained. However, it might be possible to deplete the glands to an even greater extent if the doses of acetylcholine were continued when there was no longer a release of amine sufficient to produce a vasopressor effect.

As might be expected, the rises in blood pressure produced by the acetylcholine became smaller as the doses were repeated. This was due to the liberation of less amine by each dose of acetylcholine and not to a reduction in the sensitivity to adrenaline or noradrenaline. After the response to the same dose of acetylcholine had gradually decreased it could be increased temporarily by giving a larger dose of acetylcholine. However, when the gland was nearly exhausted this could not be done. There was no precise relationship between the dose of acetylcholine, the number of doses given, and the degree of depletion obtained. Obviously there cannot be, since

the amount of amine liberated by each dose varies from animal to animal and also as the experiment proceeds.

In three experiments the acetylcholine was injected into the central stump of the left brachial artery. It was hoped that by this route a smaller dose of acetylcholine would release as much amine as a larger dose given intravenously. A further possible advantage was that it might have avoided any fall in blood pressure, which occurred in a few experiments, due to incomplete atropinization of the animal. However, the intra-arterial route was no better than the intravenous route and it also had the disadvantage of causing a pronounced contracture of the muscles of the chest and left fore-limb.

After double adrenalectomy, the dose of acetylcholine had to be increased many times to produce even a small rise in blood pressure. This indicated that the doses that were used to cause depletion of the glands were exerting their effect mainly on the adrenal medulla. The small rise occasionally seen after bilateral adrenalectomy, when a large dose of acetylcholine was given, was probably due to an effect on the sympathetic chain or on accessory chromaffin tissue. That there was very little or no loss of amine from the gland during its removal was shown by the absence of any rise in blood pressure during this time.

In Series 1 either the right or the left gland was used as a control, but in Series 2 the right gland was always the control, due to the difficulty of collecting blood from this gland. Obviously it would have been preferable to have taken control glands from either side in the latter series of experiments. However, Butterworth and Mann (1957a) have shown that the left and right glands of each cat contain the same total amount of amine and the same proportion of adrenaline and noradrenaline, irrespective of any difference in the weights of the two glands. Hence, any bias in these experiments was not considered to be a disadvantage.

From the recorded rises in blood pressure in Series 1 it was obvious that much amine was liberated during the course of each experiment. Although assay of the glands showed a depletion of catechol amines it was possible that some synthesis was taking place while the amines were being liberated. This was investigated in Series 2, where the amounts of amines released in the blood stream were compared with the loss from the gland. In these results the total amount of catechol amines was equal to or less than that lost from the gland. Therefore it was inferred that no synthesis of adrenaline or noradrenaline was taking place while there was a liberation of amines. An alternative interpretation of the results of this series is that synthesis was occurring concurrently with the depletion and that this was masked by only a % of the amines being recovered in the adrenal venous effluent. This opinion is not held by the authors, since when there was a "leak" of amines into the general circulation, as in cat Nos. 23, 26, and 27, the amine content of the adrenal blood was appreciably less than the loss from the gland. Also, if such a synthesis was occurring under the conditions of these experiments, it must have taken place at such a rate as to replace similar % of adrenaline and noradrenaline. This is unlikely in view of the subsequent observations of Butterworth and Mann (1957b). Nevertheless, it may well be that under certain conditions—such as stimulation of the splanchnic nerve, as shown by Holland and Schümann (1956)—the rate of formation is considerably increased. These workers suggest that "the chemical processes in the adrenal gland which underlie the formation of noradrenaline can be greatly accelerated by the stimulation of the splanchnic nerves, and that statements concerning rates of formation in conditions in which there is no stimulation may be inapplicable during splanchnic activity."

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References

Brücke, F., Kaindl, F., and Mayer, H. (1952). *Arch. int. Pharmacodyn.*, **88**, 407.
Bülbring, E. (1949). *Brit. J. Pharmacol.*, **4**, 234.
Burn, J. H., Hutcheon, D. E., and Parker, R. H. O. (1950). Ibid., **5**, 417.
Butterworth, K. R., and Mann, M. (1957a). *J. Physiol.*, **136**, 294.
—— —— (1957b). *Brit. J. Pharmacol.*, **12**, 415.
Dunér, H. (1954). *Acta physiol. scand.*, **32**, 63.
Elliott, T. R. (1912). *J. Physiol.*, **44**, 374.
Euler, U. S. v. (1950). *Ergebn. Physiol.*, **46**, 261.
—— and Folkow, B. (1953). *Arch. exp. Path. Pharmak.*, **219**, 242.
Hökfelt, B. (1951). *Acta physiol. scand.*, **25**, Suppl. 92.
Holland, W. C., and Schümann, H. J. (1956). *Brit. J. Pharmacol.*, **11**, 449.
Outschoorn, A. S. (1952). Ibid., **7**, 605.
Redgate, E. S., and Gellhorn, E. (1953). *Amer. J. Physiol.*, **174**, 475.
Shepherd, D. M., and West, G. B. (1951). *Brit. J. Pharmacol.*, **6**, 665.

Brit. J. Pharmacol. (1957), **12**, 427.

A COMPARISON OF THE LOCAL EFFECTS OF VARIOUS INTRAMUSCULAR INJECTIONS IN THE RAT

BY

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A method is described for the assessment of the local reaction produced by intramuscular injections. This method involves the histological examinations of the injection site after injection of material into the hamstring muscles of the rat. The results obtained with both aqueous and oily materials are described; the correlation of these results with clinical observations in man is discussed.

The phenomenon of muscle regeneration has attracted much study and experimental work in the past. The literature on this subject has been fully reviewed by Adams, Denny-Brown and Pearson (1953). The most recent study is that of Walton and Adams (1956), who were concerned with the differences in regenerative capacity of normal, denervated and dystrophic muscle after a standard injury. In this type of study investigators have naturally been concerned to inflict an injury as standard as possible, whether by crushing, incision, or the injection of highly injurious material such as alcoholic solutions. It is surprising, in view of the frequency with which drugs are administered by intramuscular injection, that few studies have been published on the amount and type of injury produced by substances likely to be given by intramuscular injection therapeutically. Hawking (1943, 1944, 1945) compared the effects of various salts of quinine and mepacrine on rabbit muscle, but included no standard materials as a basis of reference, while Lüthy (1955) and von Hochsteffer (1955) both quote the unpublished results of another investigator (Shallock) as the authority for various changes after the intramuscular injection of various pharmaceuticals. Beresford, Golberg, and Smith (1957) published a careful investigation of local tissues after the injection of iron formulations, but they too did not study the effects of any other commonly used injection to provide a standard of reference. A similar criticism applies to the less detailed studies of Glückert and Benoit (1952) on the effects of sulphonamide injections, while Brown, Wilder, and Schwarz (1944), although using several types of injection, were solely concerned with the effects of oily injections. Nevertheless, clinically it is evident that marked differences occur between the reactions produced locally by various agents and indeed between various formulations of the same agent. Some injections rarely give rise to pain or swelling at the injection site, while other substances produce such severe local changes as to preclude their general use in this manner. In the case of a new drug or of a new formulation of a drug intended for intramuscular injection, this aspect is important and some means of studying such local reactions must be developed. We here describe the method used in these laboratories and the results obtained with some simple solutions and with some substances commonly administered by intramuscular injection. This method was developed here some years ago by Miss J. M. Gates and Dr. E. Weston Hurst. Although it does not provide ideal material for fundamental studies on muscle regeneration, for which it is not designed, it does permit the careful comparison of the irritancy of various materials under conditions simulating those of human use.

MATERIALS AND METHODS

Preparation of Solutions

Stock Carbon Black Suspension.—A stock suspension of carbon black (Darco G.60) 1 in 50 in water for injection *B.P.* was made and sterilized by autoclaving at 10 lb. pressure for 30 min. It is essential to use only the purest available carbon black. Several examples tested by us gave severe local reactions. Darco G.60 was satisfactory in this respect.

Saline 0.9% w/v.—Saline solution (0.9% w/v sodium chloride) in water for injection *B.P.* con-

taining carbon black 1 in 600 (such that 3 ml. of final solution contained 2.75 cc. of saline solution and 0.25 cc. of stock carbon black suspension 1 in 50) was prepared and sterilized by autoclaving at 10 lb. pressure for 30 min.

Test Solution.—The solution to be tested containing carbon black 1 in 600 was prepared in the same manner as the saline solution, and sterilized by autoclaving at 10 lb. pressure for 30 min. If the material was thermo-labile, the sterile carbon black suspension 1 in 50 was added aseptically to the sterile test solution. The tests described in this paper were made either on aqueous solutions prepared in the laboratory or on material produced for sale by Imperial Chemical Industries Limited, Pharmaceutical Division, except for the oils, which were derived from various sources.

Methods of Injection and Dissection

A minimum of 5 rats is required for each test. The rats should be from the same litter and each weigh approximately 100 g. Since a few of the injections may not be fully intramuscular, but in fascial planes and subcutaneous tissue, it is our practice to use 10 rats for each test.

The hair was clipped from the skin of the thighs of the hind legs of the rats. The rat was then held as for intraperitoneal injection and the injection sites were swabbed with antiseptic solution. The posterior thigh muscle of the left leg was then injected with 0.2 cc. of sterile saline containing carbon black (1 in 600) using a sterile syringe and needle. Similarly, the muscle of the right leg was injected with 0.2 cc. of sterile test solution containing carbon black (1 in 600) using another sterile syringe fitted with the same size of needle as that used for the saline injection.

At 2, 6, 24, 48, and 72 hr. after injection, two rats were killed by means of chloroform.

For dissection, the rat was pinned out on the dissecting board in the supine position and the hind legs were again swabbed with antiseptic solution. (It has been found that swabbing facilitates dissection.) The legs were compared externally and any differences noted.

The skin was then reflected from the muscles. Again the legs were compared and any differences noted (such as inflammation, oedema, etc.).

The tendon at the knee joint was gripped with forceps and cut at a point below the forceps. The muscle was removed from the leg by cutting up between the bone and muscle and finally across the muscle above the site of injection. Transverse sections of muscle were then cut, using a new scalpel blade, until the injection site showing carbon black was located. (It was important that the sections be cut using a saw-like movement with the blade, as direct pressure of the blade on the muscle caused damage.) A suitable section showing carbon black in the muscle was then placed between two pieces of filter paper (Whatman No. 50) and immersed in Zenker-acetic acid fixative. If the injection was truly intramuscular, the carbon black appeared dull in the muscle. If the carbon black appeared shiny in the cut surface of the muscle, it was probable that the injected material was lying between the muscles, and the tissue was not suitable for histological examination. If this was the case another rat was sacrificed.

It was essential that all injection material should be sterile and given aseptically. It was also essential that great care should be taken not to damage the muscle during dissection.

The fixed muscles were processed through paraffin; 5 μ sections were cut and stained with Anderson's haematoxylin and eosin.

Results

Normal Saline, Water, and Hypertonic Saline.—As might be predicted, sterile normal saline gave rise to the least amount of local disturbance seen in this test. Two hours after the intramuscular injection of normal saline with the marker carbon there was separation of muscle bundles around the injection site; a few wisps of eosinophilic material between these muscle bundles indicated the presence during life of some oedema fluid. Along the needle track and in contact with the injected material a few muscle fibres were seen to be disrupted, and their cytoplasm to be more eosinophilic than normal. In a cross-section of such an injection site no more than 6 to 10 fibres were seen to be so affected. Even 2 hr. after the injection, a few polymorphs can be seen particularly in the region of the carbon granules, but also in the area of oedema (Fig. 1*a*). Six hours after the injection the evidence of oedema was diminished, and polymorphs were more frequent. By 24 hr. the oedema had subsided and polymorphs were present in moderate numbers around the damaged fibres and the carbon pigment. At this time active phagocytosis by mononuclear cells of the carbon and of damaged muscle fibres could be seen, and very occasional fibroblasts were also present near the injection site. At 48 hr., phagocytes had replaced polymorphs as the predominant cell in the reaction, and the majority of the necrotic muscle had been removed by their activities. By this time eosinophils were seen in moderate numbers. Fibroblastic proliferation was brisk, and the injected carbon was beginning to be surrounded by a fibrous capsule. At 72 hr. this capsule was complete; it involved also a few muscle bundles in the immediate vicinity of the carbon. The majority of the phagocytes had disappeared, the whole injection site now appeared tidy, and the reaction had subsided completely (Fig. 1*b*).

Sterile distilled water gave rise to a surprisingly severe reaction when injected intramuscularly.

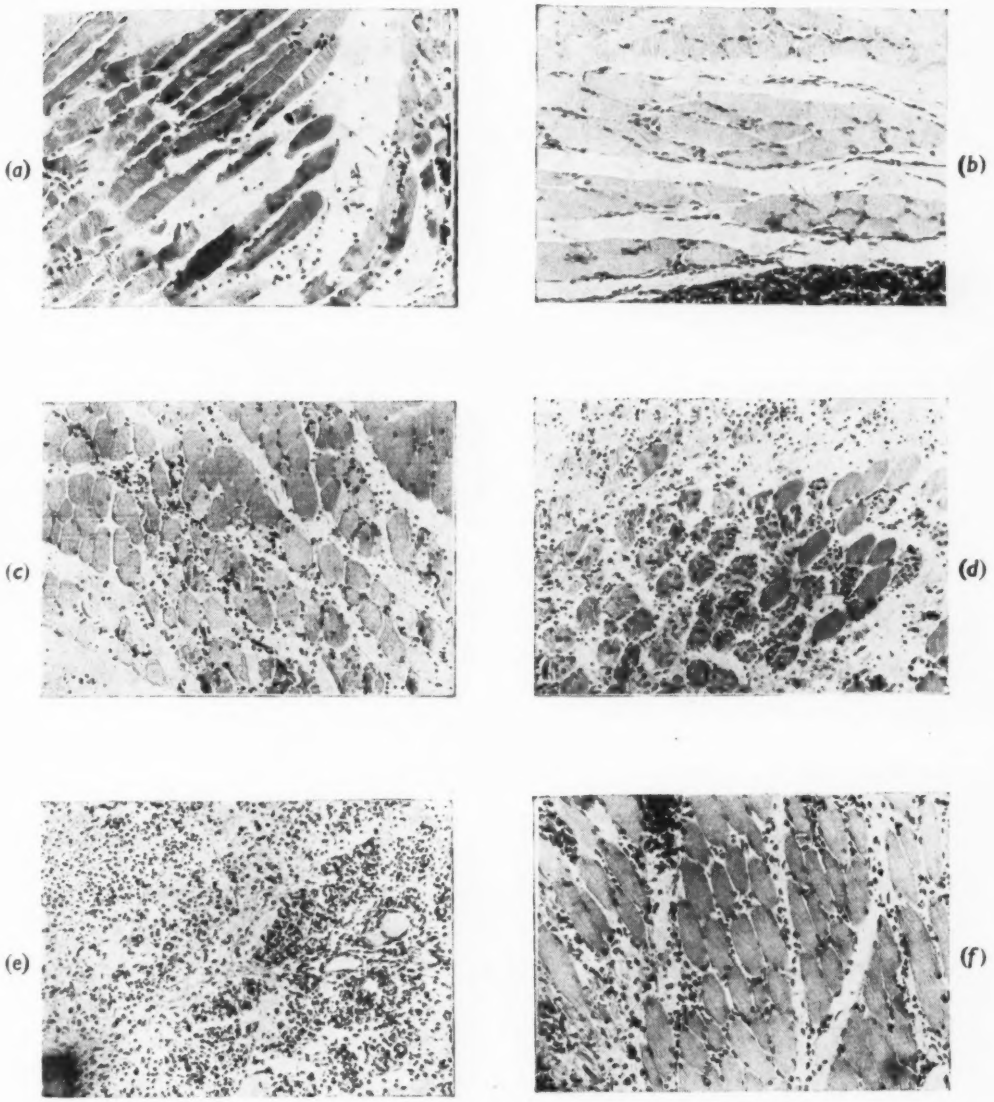

FIG. 1.—Photomicrographs of muscle fibres stained with haematoxylin and eosin from the hamstring muscles of rats receiving intramuscular injections. Magnification in all photomicrographs, × 75. The time shown in parentheses indicates the period between injection and removal of specimen for histological examination. (*a*), Isotonic saline (2 hr.). Slight oedema and polymorphonuclear infiltration close to carbon. (*b*), Isotonic saline (72 hr.). A thin fibrous capsule surrounds the carbon. (*c*), Distilled water (6 hr.). Considerable oedema and polymorphonuclear infiltration. Note the invasion of occasional necrotic muscle fibres by polymorphonuclear leucocytes. (*d*), Distilled water (24 hr.). Extensive invasion of necrotic muscle fibres by polymorphonuclear leucocytes and phagocytes. (*e*), Distilled water (72 hr.). Considerable polymorphonuclear and phagocyte activity. No damaged muscle remains unattacked. (*f*), Hypertonic saline (72 hr.). Rather extensive fine fibrosis. Compare with (*b*).

Two hours after the injection, the carbon was surrounded by a zone some 2 mm. in diameter in which the muscle bundles, and the individual fibres within the bundles, were separated by oedema. In this zone numerous fibres appeared disrupted and many other fibres showed altered staining properties, some being swollen and eosinophilic, others markedly basophilic. At the edge of this markedly oedematous zone the muscle bundles were separated by oedema, but the bundles themselves were not disrupted. At this time polymorphs were present in quite large numbers throughout the oedematous area ; others could be seen in the process of migration from vessels in the area.

Six hours after the injection the oedema had subsided somewhat, although much still remained. By this time numbers of muscle fibres throughout the affected area were swollen and obviously necrotic; in some, invasion of the cytoplasm by polymorphs had commenced (Fig. 1*c*). Occasional macrophages could now be seen. By 24 hr. the acute inflammatory reaction was fully established and the whole area around the injection site was infiltrated with polymorphs and macrophages. This infiltration was most intense around the carbon, but was marked throughout the oedematous area. Polymorph invasion and phagocytosis of damaged muscle fibres was well advanced (Fig. 1*d*). At 48 hr., polymorph infiltration was still prominent, but phagocytes had increased in number and fibroblasts were now appearing, particularly around the carbon, but also around damaged muscle fibres. Occasional basophilic muscle fibres with central nuclei were now seen. These were much more numerous at 72 hr., when large areas of such proliferating muscle were seen. At this time, fibroblastic proliferation was marked, but evidence of a continuing acute inflammatory response, with polymorph and phagocyte infiltration of the damaged area, could still be seen. Damaged and necrotic muscle fibres were still present although in small numbers, and active phagocytosis of these was proceeding (Fig. 1*e*). Sections from animals in which the injection had not been fully intramuscular demonstrated that a similar intense reaction occurred in the subcutaneous adipose and connective tissue.

By contrast, hypertonic saline (1.7%) gave rise to little more damage to muscle than did isotonic saline. However, the oedema which arose was much more extensive and separated muscle bundles quite remote from the injection site, although the individual fibres in these bundles were not damaged. Inflammatory reaction appeared early, and extended throughout the oedematous area; this area was also involved in the ultimate fibrosis (Fig. 1*f*).

Calcium Gluconate.—Calcium gluconate 10% gave rise to little more tissue damage at the injection site than did saline. About the same number of fibres were necrotic: rather more showed swelling and other signs of damage short of actual necrosis. The cellular response was greater in amount at all times after the injection of calcium gluconate than after the injection of saline; 2 hr. after the injection quite large numbers of polymorphs were seen at the injection site.

Potassium Penicillin.—The injection of potassium penicillin 500,000 units/ml. gave rise to a zone of oedema 3 to 4 mm. diameter at 2 hr. In this zone all the muscle fibres appeared swollen, their cytoplasm was homogeneous and eosinophilic and the nuclei pyknotic. No polymorphs or other cellular reaction could be seen 2 or 6 hr. after the injection (Fig. 2*a*). By 24 hr. small numbers of polymorphs could be seen at the periphery of the damaged area and some also around the carbon at the centre. There was, however, no general invasion of the oedematous area, or of the necrotic muscle, by polymorphs or histiocytes. At 72 hr. this area was still devoid of polymorphs or other cells, although at this time the polymorph reaction around the central carbon was brisk, and at the periphery granulation tissue formation and muscle proliferation were proceeding as well. In some sections at all periods after the injection some damage to blood vessels could be seen, although this was not usually of severe degree.

The injection of potassium penicillin at a strength of 100,000 units/ml., at which strength the solution is isotonic, gave rise to an area of damaged muscle scarcely less than that caused by the stronger solution, nor did the character of the reaction differ appreciably.

Quinine Dihydrochloride.—Two hours after the injection of quinine dihydrochloride the damaged area was 6 to 8 mm. in diameter. Oedema was most marked at the periphery of this zone; at its centre the muscle fibres appeared to have been fixed before separation by oedema fluid could occur. In many sections some haemorrhage could be seen in the central parts of the lesion, and the red cells in some venules appeared damaged, while other veins showed damaged walls. After the injection of quinine dihydrochloride a large proportion of the damaged muscle fibres was strongly basophilic. Cellular reaction at the injection site was first seen 24 hr. after the injection, when it was confined to a sparse polymorph infiltration at the extreme periphery of the damaged area. Even 72 hr. after the injection, there was no inflammatory infiltration of the main bulk of damaged muscle, nor was any reaction to be seen surrounding the carbon (Fig. 2*b*). At this time there was a brisk inflammatory infiltration at the periphery of the damaged area, with muscle proliferation, granulation tissue formation and commencing fibrosis (Fig. 2*c*).

Oily Injections

The agents described above were all aqueous solutions. Non-aqueous injections, as might be expected, produced a different type of reaction at the site of injection. Two examples of this type of reaction are described below.

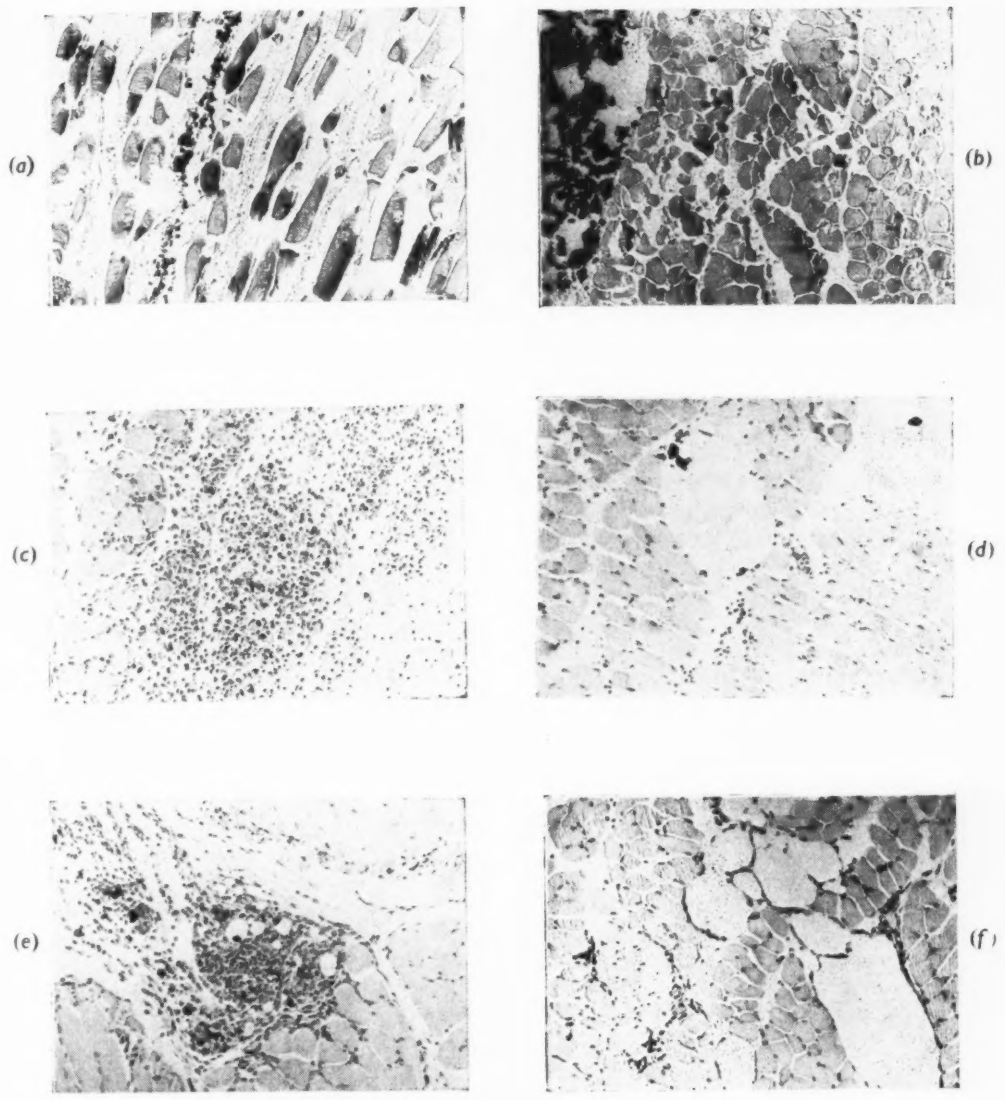

FIG. 2.—Photomicrographs as in Fig. 1. (*a*), Potassium penicillin (6 hr.). Severe muscle damage, no cellular reaction. (*b*), Quinine dihydrochloride (72 hr.). Central portion of lesion showing necrotic muscle devoid of cellular reaction. (*c*), Quinine dihydrochloride (72 hr.). Edge of lesion showing phagocytosis of damaged muscle fibres and fibrosis proceeding simultaneously. (*d*), Liquid paraffin (6 hr.). Slight cellular reaction around oil droplets; no damaged muscle fibres present. (*e*), Liquid paraffin (72 hr.). A considerable granuloma, in which eosinophil cells are prominent, has formed around the oil droplets. (*f*), Maize oil (72 hr.). Only a slight reaction to the oil droplets is seen.

Two hours after the injection of liquid paraffin little damage had occurred. The muscles surrounding the needle track appeared to have been pushed aside by the injected material, but only isolated fibres showed evidence of damage, presumably by trauma from the needle. There was no zone of oedema, and no evidence of cellular reaction. By 6 hr. rather more fibres showed evidence of damage, but it was still not extensive. The injection had now split into numerous isolated globules, which lay between, and distorted, individual muscle bundles without greatly damaging them. The oil droplets were surrounded by a little reaction with histiocytes and polymorphs in almost equal numbers (Fig. 2*d*). In these sections, and indeed in all the later sections from sites of injection of oily substances, it could be seen that there was a considerable tendency for the injec-

tion fluid to leak back along the needle track and into the subcutaneous tissues.

The muscle damage did not appear to increase during the next three days. The isolated globules of oil become well defined, by reason of a cuff of histiocytes and fibroblasts surrounding each individual globule. Even at 24 hr., polymorphs were no longer prominent in the cellular response; their place was taken apparently by eosinophils, which may be quite prominent in these granulomata. This prominence of eosinophils appeared especially marked in granulomata formed around globules of oil which had leaked back into the subcutaneous tissues. At 72 hr. each of these granulomata had, in addition to the eosinophils and histiocytes, a well-marked fibrous capsule (Fig. 2*e*).

The injection of maize oil gave rise to slightly more damage to muscle than did the injection of liquid paraffin, although the amount was still small compared with that found following the injection of even the less injurious aqueous solutions. The cellular response to maize oil was of the same general character as that to liquid paraffin, but it was much smaller in amount, nor was fibrosis at all prominent even at 72 hr. At this time, the individual globules were separated only by thin capsules consisting of a few fibroblasts with eosinophils and histiocytes (Fig. 2*f*).

Discussion

In man, purely local reactions to injected material may take the form of pain occurring immediately upon injection of the material, or very shortly thereafter, or of inflammatory reactions, sometimes with abscess formation occurring during the days following the injection. More rarely reactions remote in time from the injection may occur, for example the paraffin granuloma.

The mechanism of pain occurring immediately on injection is not clear. A possible explanation is that some substances may possess the property of causing excitation of nerve fibres and nerve endings with which they come into contact. Whatever the mechanism, no animal experiment to determine the probability of this event occurring in man readily suggests itself, and trial in man appears to be the only way of discovering whether this complication will occur.

If contamination by pathogenic organisms be excluded, inflammatory reactions occurring at an injection site must be due to the local effects of the injected material. Since, as has been shown above, even the most bland injection will cause some local damage, while local reactions sufficient to evoke clinical comment are not common with most injectable preparations, it is clear that a wide latitude exists in the damage that may occur at an injection site before a clinically evident condition manifests itself. This is fortunate, for, were this not so, therapy by intramuscular injection would be virtually impossible. It remains therefore to decide whether any parallel exists between effects produced by a substance in rat muscle on injection and the clinical effects of the same substance in man, and, if such a parallel exists, what amount of damage in the rat muscle represents the upper limit of clinical acceptability in man. Here it must be pointed out that clinical acceptability is not absolute, but depends on the purpose and efficacy of the medication; any local reaction no matter how severe would be acceptable in a reliable cure for cancer, while no reaction at all would be permissible in a cure for the common cold.

It is immediately clear that at least a rough parallel does exist between the rat test and results in man. Substances known to be bland on injection in man produce minimal local damage when injected into rat muscle. Normal saline and calcium gluconate exemplify this concordance. However, when substances differ markedly in the reaction produced in rat muscle as a consequence of their injection from the reaction consequent on the injection of saline there are no general grounds for predicting what level of reaction in rat muscle represents a clinically undesirable reaction even if the parallel between the two situations is always valid.

We have resolved this difficulty by the observation that all the substances known regularly to give rise to unfavourable clinical reactions, when tested in the rat, give rise to such necrosis of muscle that at 72 hr. necrotic muscle is still to be seen around which no reaction is occurring. It may also be that the frequency and severity of the clinical reactions are roughly related to the amount of such necrotic muscle. Quinine dihydrochloride is described above as an example of such a noxious injection. We have also found that, as might be expected, such substances give other evidence of their noxious properties. Thus, oedema is severe when necrosis is extensive, and the appearance of inflammatory cells is much delayed. Although this observation is empirical, it may perhaps be related to clinical experience more directly, since necrotic muscle present at three days, and not subject to phagocytic attack, must form a potential site for a sterile abscess due to autolysis of the dead muscle and the accumulation of necrotic inflammatory exudate, while dead

muscle fully phagocytosed in the first three days obviously does not represent so severe a hazard in this respect. It is worth noting that such residual dead muscle may also form a suitable nidus for bacterial growth.

It is thus our experience that, by this test, injectable materials can be divided into three categories: (*a*) Substances causing little more damage than normal saline. These may be expected to prove bland on injection in man. (*b*) Substances which in this test give a residue of necrotic muscle unattacked by phagocytes at 72 hr. These may be expected, in man, to give rise to severe local reactions at the injection site, often sufficient to preclude their use. (*c*) Substances giving rise to damage intermediate between (*a*) and (*b*). Prediction here is less certain, but examination of the histological appearances may suggest that the picture is close to one or other groups above, and the probability of clinical reaction may be correspondingly assessed.

Several factors must influence the local irritancy of injected materials, even when these are single pure substances in simple solution. Often the substance itself cannot, for therapeutic reasons, be appreciably modified. Our observations on distilled water, normal saline and hypertonic saline show that the tonicity of a solution modifies very strikingly the resultant muscle reaction. It is thus always advisable for injectable formulations to be as close to isotonicity as possible. This is not always efficacious in diminishing irritancy, as the observations on potassium penicillin demonstrate. This solution is approximately isotonic at 100,000 units/ml., but the irritancy of such a solution differs little from that of a solution of 500,000 units/ml.

Oils and oily solutions present a somewhat different problem from those discussed above. Few oils produce the immediate severe muscle damage seen with some aqueous solutions; however, the formation of a chronic progressive granuloma is a hazard, and this can be assessed equally well by the test we describe. We find it advisable to supplement the routine test with observations made at later periods following the injection. Nevertheless, even at 72 hr. it can be seen that the various oil granulomata differ greatly in size and activity. Our results are in agreement with those of Brown *et al.* (1944) in this respect and also when responses to vegetable oils are considered. A striking and constant feature of the oil granulomata seen in our rats with the oils described above, and with others not described here, is the eosinophil reaction. Eosinophils were noted by Story (1950) in an oil granuloma in man, although eosinophils were not noted in their animals by Brown *et al.* (1944). The significance of this phenomenon is not clear. It does not appear to be related to sensitization, and it can be evoked by oils of very diverse compositions.

Ultimately the only valid criterion of the clinical acceptability of an injection is, of course, trial in man. Before such a trial, it is desirable to have some idea of the likely behaviour of the material. Although many simple procedures could be developed to examine some aspect of the local irritancy of substances, none, we think, would model so closely conditions of use, and none would provide so reliable a basis for prediction of effects in man, as the technique described above.

References

Adams, R. D., Denny-Brown, D., and Pearson, C. M. (1953). *Diseases of Muscle*, 1st ed. New York: Boeber.

Beresford, C. R., Golberg, L., and Smith, J. P. (1957). *Brit. J. Pharmacol.*, **12**, 107.

Brown, W. E., Wilder, Violet M., and Schwarz, Pauline (1944). *J. Lab. clin. Med.*, **29**, 259.

Glückert, H., and Benoit, W. (1952). *Nervenartz*, **23**, 17.

Hawking, F. (1943). *Brit. med. J.*, **2**, 198.

—— (1944). Ibid., **2**, 209.

—— (1945). Ibid., **1**, 412.

Hochsteffer, A. von (1955). *Schweiz. med. Wschr.*, **85**, 1138.

Lüthy, F. (1955). Ibid., **85**, 1092.

Story, P. (1950). *Brit. med. J.*, **1**, 1467.

Walton, J. N., and Adams, R. D. (1956). *J. Path. Bact.*, **72**, 273.

Brit. J. Pharmacol. (1957), **12**, 434.

THE ANTITUBERCULOUS ACTIVITY OF ETHYL THIOLESTERS WITH PARTICULAR REFERENCE TO DIETHYL DITHIOL*ISO*PHTHALATE

BY

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Diethyl dithiol*iso*phthalate exerts an antituberculous effect in mice comparable to that of isoniazid and streptomycin. It is effective when given in single doses, and against isoniazid-resistant strains. It is most active when given subcutaneously either by injection or by absorption through the skin. Drug resistance has been demonstrated in mice, and isoniazid is active against such strains. The effects of this and other ethyl thiolesters are antagonized by their methyl analogues.

In a previous paper (Davies, Driver, Hoggarth, Martin, Paige, Rose, and Wilson, 1956) it was suggested that the thiol esters were the most promising of the antituberculous derivatives of ethyl mercaptan. Since the completion of this work Solotorovsky, Winsten, Ironson, and Brown (1956) have described the antituberculous activity of ethyl mercaptan derivatives including thiol esters.

We describe here experiments in mice with diethyl dithiol*iso*phthalate (ETIP). This compound was selected for study in mice because it appeared to be the most suitable of the series for clinical trial in man.

METHODS

Male albino mice weighing 18 to 22 g. were infected intravenously with 0.5 mg. wet weight of *Mycobacterium tuberculosis*, human type, strain 905, suspended in 0.1 ml. of distilled water. The suspension was made by ball-milling bacilli harvested from a 14-day culture on Löwenstein's medium. Ethyl thiolesters were given subcutaneously to mice as solutions in arachis oil.

RESULTS

Acute Infections.—Table I shows the results of the first experiment in which ETIP was used in mice. ETIP showed high activity, and subcutaneous injections were more effective than doses given by mouth. Animals which received 500 mg./kg. of ETIP subcutaneously each day for 10 days were all alive 133 days after infection, while the untreated control mice had a mean survival time of 16.9 days.

The most active derivative of ethyl mercaptan which we had tested in mice before the discovery of ETIP was diethyl dithiolcarbonate. In the direct comparison shown in Table II, ETIP was as active as diethyl dithiolcarbonate at high doses, but less active at low doses. ETIP is virtually odourless, whereas the very unpleasant odour of diethyl dithiolcarbonate makes it unsuitable as a substance for use in man.

TABLE I

THE EFFECT OF DIETHYL DITHIOL*ISO*PHTHALATE (ETIP) AGAINST A TUBERCULOUS INFECTION IN MICE

Mice treated for 2 weeks, each of 5 days, starting from the day of of infection. 10 mice/group.

Dose of ETIP (mg./kg.)	Mean Survival Time (Days)
1,000 (oral)	63·7
500 ,,	25·8
500 (subcut.)	133 (no mice dead on the 133rd day)
None	16·9

TABLE II

COMPARISON OF ETIP WITH DIETHYL DITHIOLCARBONATE

Groups of 10 mice dosed for 2 weeks each of 5 days from day of infection.

Dose (mg./kg., subcut.)	Mean Survival Time (Days)	
	ETIP	Diethyl Dithiolcarbonate
250	80·0	69·7
125	50·2	59·2
50	36·0	80·7
25	23·2	42·2
12·5	21·0	35·3
5	21·4	28·4
None	21·6	

It was observed that application of ETIP to intact human skin resulted in an odour of ethyl mercaptan in the breath. This clearly indicated absorption of the drug through the skin and suggested that local application might give effects equivalent to subcutaneous administration. Groups of infected mice were treated by the daily application to the plucked skin for 10 days of a paste containing 75% ETIP and 25% magnesium stearate. The amount of paste applied contained in each dose the equivalent of 10 mg./kg. ETIP. To ensure that the effect was not due to mice licking the drug from one another, in a second experiment the mice were isolated in individual cages and watched for 1 hr. after application, when absorption appeared to be complete. The animals made no attempt to lick off the drug. In both these experiments a marked increase in the survival time (31 and 36 days respectively) was observed in the treated groups.

ETIP was also highly active against an intracorneal infection in mice (Table III). Naguib and Robson (1956) have shown it to be active in intracorneal infections with murine leprosy in mice.

TABLE III

THE EFFECT OF ETIP AND ISONIAZID ON AN INTRACORNEAL INFECTION IN MICE

Groups of 10 mice treated for 1 month from day of infection.

Treatment	Dose (mg./kg., by Mouth)	Number of Eyes Showing Lesions					
		Days after Infection					
		28	37	50	64	78	114
Isoniazid ..	100	1	1	4	5	5	6
ETIP ..	1,000	1	3	3	3	3	4
None ..	—	9	10	10	10	10	10

When single doses of 500 mg./kg. of ETIP were given subcutaneously to groups of 10 mice at various times before and after infection, activity was demonstrated with the compound given either 48 hr. before or 7 days after infection.

The Established Infection.—The compound was also markedly active against an established infection. The effect produced by daily subcutaneous doses of 500 mg./kg. of ETIP for 10 days was comparable to that produced by 50 mg./kg. of isoniazid given orally twice daily for 10 days. The 500 mg./kg. dose of ETIP, given twice daily orally, was as active as 100 mg./kg. of streptomycin, given subcutaneously once daily. The effect of ETIP appeared to fall off rapidly at doses below 250 mg./kg./day. The mean survival times in days of the groups in this experiment were: ETIP (500 mg./kg.) 78.7: ETIP (250 mg./kg.) 60.3: ETIP (125 mg./kg.) 19.3: streptomycin (100 mg./kg.) 35.5: isoniazid (50 mg./kg. twice daily) 76.3: controls 18.1.

Effect of Weekly Doses of ETIP.—A marked antituberculous effect was obtained with a single subcutaneous dose of 500 mg./kg. of ETIP given on the day of infection, and still larger effects were obtained by giving one or more further doses at weekly intervals (Table IV). One group of mice was dosed once weekly until death and the mean survival time of these animals was 105.3 days compared with the mean survival time of 19.3 days in the untreated group. Isoniazid was less active under these very stringent conditions.

TABLE IV

THE EFFECT OF ETIP AND ISONIAZID WHEN GIVEN AS SINGLE DOSES AT WEEKLY INTERVALS TO GROUPS OF 10 MICE

Treatment	Dose (mg./kg., subcut.)	Mean Survival Time (Days)
ETIP on day 0 only	500	28·1
,, ,, days 0 and 7 ..	500	55·5
,, ,, ,, 0, 7, and 14 ..	500	64·0
,, once weekly until death	500	105·3
Isoniazid on day 0 only ..	50	21·7
,, ,, days 0 and 7 ..	50	40·2
,, ,, ,, 0, 7, and 14	50	40·2
,, once weekly until death	50	94·2
None	—	19·3

Experiments with ETIP and Isoniazid.—As combined treatment is now well established in the therapy of tuberculosis, the effect of combinations of isoniazid and ETIP was studied. Table V shows that the combination of the two drugs was much better than either alone.

TABLE V

THE ADDITIVE EFFECT OF ETIP AND ISONIAZID

Isoniazid was incorporated in the food; the dose of ETIP was given subcutaneously. An asterisk indicates that these animals died from causes other than tuberculosis.

Treatment	Dose (mg./kg.)	Mean Survival Time (Days)
None ..	—	18·5
Isoniazid ..	8·5	49·3
ETIP ..	125	50·5
ETIP ..	50	24·7
		Days of Death of Individual Mice
Isoniazid + ETIP	7·5 125	*9, *29, 40, 69, 80, 100, 100, +3 survivors on 100th day
Isoniazid + ETIP	7·5 50	59, 69, 69, 73, +6 survivors on 100th day

A single dose of ETIP markedly prolonged the life of infected mice that had relapsed and were about to die after treatment with isoniazid. Treatment was effective even when the ETIP was given

only 5 days before mice in a group which had been treated with isoniazid alone started to die (Table VI).

TABLE VI

THE EFFECT OF SINGLE DOSES OF ETIP GIVEN TO INFECTED MICE AFTER A COURSE OF TREATMENT WITH ISONIAZID

All mice except the untreated controls were given isoniazid (7·5 mg./kg. in food) for the first 10 days after infection. S indicates a mouse which was alive 161 days and A 63 days after infection. K indicates that the mouse was killed.

Dose of ETIP (mg./kg.)	Days of Death of Individual Mice	Mean Survival Time (Days)
None ..	24, 30, 30, 36, 41, 41, 46, 84, K119, K119	57·0
500 (on day of infection)	36, 38, 50, 71, 90, 119, 119, 121, 126, 127	89·7
500 (20 days after) ..	71, 75, K119, K119, 133, S, S, S, S, S	132·2
500 (25 days after) ..	61, 83, 83, 97, K119, K119, 124, S, S, S	116·9
500 (30 days after) ..	27, 30, 31, 47, 77, 110, K119, K119, 146, 160	86·6
Untreated controls	18, 19, 20, 21, 21, 22, 22, 23, 23, 23	21·2
None ..	25, 26, 28, 28, 31, 32, 35, 49, A	34·8
50 (on day of infection)	23, 24, 25, 26, 28, 30, 33, 43, A, A	35·8
50 (20 days after) ..	26, 28, 59, A, A, A, A, A, A, A	55·4
50 (25 days after) ..	27, 28, 28, 28, 28, 29, 30, 30, 49, A	34·0
Untreated controls	18, 18, 18, 19, 20, 20, 20, 20, 21, 21	19·5

Drug Resistance.—One of the main needs in the present-day chemotherapy of tuberculosis is for a non-toxic substance, antituberculous in its own right, which will act upon tubercle bacilli that are resistant to isoniazid. Whatever the origin of such resistant strains, whether they are adaptive forms produced in response to the drug or spontaneous mutants, their emergence will probably be delayed in the presence of a second drug to which no cross-resistance is shown. We have demonstrated that there is no cross-resistance between isoniazid and ETIP. Mice infected with tubercle bacilli resistant to isoniazid respond to treatment with ETIP. The effect of ETIP on a streptomycin-resistant strain was not tested, since in an earlier experiment we had found that other ethyl mercaptan derivatives such as ethyl thiolbenzoate were effective against infections caused by such strains. An important factor governing the use of drugs in the treatment of tuberculosis is the ease with which tubercle bacilli become drug-resistant. All the commonly-used therapeutic agents (streptomycin, isoniazid, PAS) are active against tubercle bacilli *in vitro* and resistance to them can be demonstrated by *in vitro* tests. We have already shown (Davies *et al.*, 1956) that the slight *in vitro* activity of the compounds derived from ethyl mercaptan is probably irrelevant to their therapeutic action. Development of drug resistance, therefore, had to be studied *in vivo*. A group of 10 mice were infected and treated for 10 days with daily subcutaneous doses of ETIP at the rate of 250 mg./kg. On the 11th day after infection 5 mice were killed and the tubercle bacilli from their lungs cultured on Löwenstein's medium. After incubation for one month, these cultures were mixed and used to infect 2 further groups of mice, one of which was treated as before with ETIP, and the other left untreated. Resistance to ETIP was then complete, since a dose of 250 mg./kg. had no therapeutic effect and resistance once developed appeared to be fairly permanent, one culture being still resistant after seven monthly passages on Löwenstein's medium in the absence of drug. This strain was also resistant to other thiolesters. Isoniazid, however, was fully effective against the ETIP-resistant strain.

Antagonism.—A possible mode of action of ethyl mercaptan is that it interferes with a biological methylating or thiomethylating system. In support of this hypothesis it was found that the activity of sodium S-ethyl thiosulphate was antagonized by concurrent administration of sodium S-methyl thiosulphate. Similarly, ethyl thiolbenzoate, diethyldisulphide and diethyl dithio*iso*phthalate were antagonized by methyl thiolbenzoate. The specificity of this action was demonstrated by the failure of methyl thiolbenzoate to antagonize the action of isoniazid and the failure of propyl- and *iso*propyl thiolbenzoates to antagonize ethyl thiolbenzoate. However, dimethyldisulphide did not antagonize either diethyldisulphide or ethyl thiolbenzoate, and ethyl benzoate slightly antagonized ethyl thiobenzoate and ETIP but not isoniazid (Table VII). Ethyl thiolbenzoate was not antagonized by cystine, cysteine, methionine or glutathione.

The experiments reported in this paper show that diethyl dithio*iso*phthalate is a potent antituberculous agent in mice. Its high activity was apparent from the effect of single doses. Repeated subcutaneous administration resulted in a very marked increase in the survival time of infected mice (Table I), but tubercle bacilli were still recoverable from animals surviving for 133 days. This recovery could be accounted for in one of two ways: either the drug had failed to sterilize, or the mice had become reinfected. In unpublished experiments we have shown that this second possibility is real since normal mice become infected with tuberculosis when they are kept near diseased mice for several weeks.

TABLE VII

ANTAGONISM OF ANTITUBERCULOUS ACTION OF DERIVATIVES OF ETHYL MERCAPTAN

All treatments were started on the day of infection and continued for 10 days. Therapeutic effect is defined as the difference in days between the mean survival times of treated and control groups.

Active Agent	Daily Dose (mg./kg.)	Additive	Daily Dose (mg./kg.)	Therapeutic Effect (Days)		
				With Additive	Without Additive	Difference
Ethyl thiosulphate	500 oral	Methyl thiosulphate	500 oral	−0·6	5·6	6·2
Ethyl thiolbenzoate	250 s.c.	Methyl thiolbenzoate	250 s.c.	0·3	26·1	26·4
ETIP	250 ,,	,, ,,	250 ,,	10·2	33·2	23·0
Dimethyldisulphide	125 ,,	,, ,,	250 ,,	5·1	20·0	14·9
Isoniazid	25 oral	,, ,,	250 ,,	33·3	29·8	−0·5
Ethyl thiolbenzoate	250 s.c.	*n*-Propyl thiobenzoate	250 ,,	22 6	30·6	8·0
,,	250 ,,	*iso*-Propyl thiolbenzoate	250 ,,	31·9	30·6	1·3
Diethyldisulphide	125 ,,	Dimethyldisulphide	125 ,,	23·9	28·2	4·3
Ethyl thiolbenzoate	250 ,,	,,	125 ,,	26·0	25	−1·0
Diethyldisulphide	125 ,,	Di-*n*-propyl disulphide	125 ,,	26·2	28·2	2·0
,,	125 ,,	Di-*iso*-propyl disulphide	125 ,,	32·0	28·2	−3·8
Ethyl thiolbenzoate	250 ,,	Ethyl benzoate	250 ,,	10·4	26·1	15·7
ETIP	250 ,,	,, ,,	25 ,,	18·8	33·2	14·4

The percutaneous activity of ETIP is a novel observation which suggests a method of administration suitable for use in man since pastes prepared from the drug appear to be stable and non-irritant.

Inclusion of isoniazid and ETIP in the same experiments showed that the two drugs were compatible and there was no evidence of cross-resistance. Of particular interest are the results shown in Table VI in which mice were allowed to relapse after treatment with isoniazid and then given single doses of ETIP; moribund mice recovered and lived for a further 100 days. The effect of weekly doses of isoniazid or of ETIP suggests that in mice it is not necessary to maintain constant high blood levels with these drugs. It should be pointed out, however, that these results may apply only to mice and the experiment is not regarded as providing sufficient evidence for the use of a similar dosage schedule in man.

The development of drug-resistance suggests that at least part of the action is exerted directly on the bacteria and is not mediated solely through the defence mechanisms of the host. Antagonism of ethyl thiol compounds by their methyl-analogues may indicate that ethyl mercaptan interferes with an essential methyl- or methylthiol-containing system. A number of such systems were investigated and reported in a previous paper (Davies *et al.*, 1956) and it may be that a hitherto unknown biological system is involved. However, dimethyldisulphide does not antagonize the action of ethyl thiol compounds. The metabolism of dimethyldisulphide may differ from that of diethyldisulphide, methyl mercaptan and the other derivatives of methyl mercaptan.

The partial antagonism of ethyl thiolbenzoate and ETIP by ethyl benzoate is probably explained by competition of the benzoate with the thiol esters for available esterase activity resulting in a reduced liberation of ethyl mercaptan. Isoniazid was not antagonized by ethyl benzoate.

REFERENCES

Davies, G. E., Driver, G. W., Hoggarth, E., Martin, A. R., Paige, M. F. C., Rose, F. L., and Wilson, B. R. (1956). *Brit. J. Pharmacol.*, **11**, 351.

Naguib, M., and Robson, J. M. (1956). *Lancet*, **1**, 411.

Solotorovsky, M., Winsten, S., Ironson, E. J., and Brown, H. D. (1956). *Amer. Rev. Tuberc.*, **74**, 59.

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PROTECTION AGAINST THE LETHAL EFFECTS OF ORGANOPHOSPHATES BY PYRIDINE-2-ALDOXIME METHIODIDE

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The mechanism responsible for the protection against lethal organophosphate poisoning by pyridine-2-aldoxime methiodide (P-2-AM) was studied in the mouse. Two types of organophosphates were used: ethyl pyrophosphate (TEPP), E 600, Ro 3-0340, and Ro 3-0422 which form with true cholinesterase a diethylphosphoryl enzyme (1) and DFP, D 600, and Ro 3-0351 which form with true cholinesterase a di*iso*propylphosphoryl enzyme (2).

In vitro and under the experimental conditions used more than 50% reactivation of (1) was obtained within 1 hr. by concentrations of P-2-AM ranging from 0.5 to 1×10^{-5} M; 30 times higher concentrations of the oxime were required to achieve the same effect with (2). *In vivo* reactivation of phosphorylated true cholinesterases in blood amounted to 10 to 24% within the first 30 min. if 25 mg./kg. P-2-AM was injected (i.p.) 5 min. before a sublethal dose of TEPP, E 600, Ro 3-0340, or Ro 3-0422 and reactivation reached a maximum within 1 to 2 hr. after the injection of the oxime. P-2-AM was more effective when given 30 min. after the organophosphate. The effect of 25 mg./kg. P-2-AM on the phosphorylated true cholinesterase in brain (experiments with TEPP and E 600) was negligible. A dose of 25 mg./kg. P-2-AM had no consistent effect on the phosphorylated true cholinesterases in blood and brain of mice injected with sublethal doses of DFP, D 600, or Ro 3-0351.

The protection by 25 mg./kg. P-2-AM against lethal doses of TEPP, E 600, Ro 3-0422, and Ro 3-0340 was greater than that obtained with 50 mg./kg. atropine sulphate, but the degree of protection was determined by the organophosphate itself and not its dialkylphosphoryl group. Protection by 25 mg./kg. P-2-AM against lethal doses of DFP, D 600, and Ro 3-0351 was negligible. The antidotal effect of P-2-AM was potentiated by atropine. Mice which were injected with atropine and P-2-AM were protected to a greater extent against DFP than against Ro 3-0422, and protection against DFP was only slightly less than protection against TEPP. This is difficult to reconcile with a specific action of P-2-AM on phosphorylated cholinesterases.

The interaction between cholinesterase and organophosphates which have a common dialkylphosphoryl group leads to the formation of an inhibited enzyme with identical properties (Burgen and Hobbiger, 1951; Aldridge and Davison, 1953; Hobbiger, 1954, 1955 and 1956). The reason for this is that cholinesterase attempts to hydrolyse the organophosphates, but the hydrolysis process is incomplete and the dialkylphosphoryl group of the organophosphate—unlike the acyl group of a true substrate—remains attached to the enzyme for a considerable period. Reactivation of phosphorylated cholinesterase in aqueous solution is determined by the dialkylphosphoryl group and can be markedly enhanced *in vitro* by nucleophilic reagents which remove the dialkylphosphoryl group from the enzyme by virtue of their high affinity for the phosphorus. One of the most powerful reactivators of phosphorylated true cholinesterase is pyridine-2-aldoxime methiodide (P-2-AM) (Childs, Davies, Green, and Rutland, 1955; Wilson and Ginsburg, 1955). The concentrations of P-2-AM which are required to restore 50% of the activity of phosphorylated human true cholinesterase within 1 hr. at 37° and *p*H 7.4 (medium: 0.025 M $NaHCO_3$, 0.075 M NaCl, 0.075 M KCl, 0.04 M $MgCl_2$ and 0.1% bovine plasma albumin) are 2.7×10^{-5} M for the diethylphosphoryl enzyme and 6.1×10^{-4} M for the di*iso*propylphosphoryl enzyme (Hobbiger, 1956). Thus it seems possible that P-2-AM and (or) other oximes might be of therapeutic value in organophosphate poisoning. To study this, experiments were carried out in mice, and the following account gives the results obtained on the reactivation *in vitro* of phosphorylated true cholinesterases

in blood and brain by P-2-AM, the effect of P-2-AM *in vivo* on the same enzymes and the protective action of P-2-AM against lethal organophosphate poisoning. Additional results show that in some cases atropine markedly potentiates the antidotal effect of P-2-AM in organophosphate poisoning. The degree of protection achieved by P-2-AM and by the combined use of atropine and P-2-AM is determined by the organophosphate itself and not its dialkylphosphoryl group, and results obtained with an individual organophosphate (Kewitz, Wilson, and Nachmansohn, 1956) are, therefore, not applicable to all organophosphates which form the same type of phosphorylated enzyme.

METHODS

Male white mice weighing between 18 and 20 g. were used in all experiments. The organophosphates were injected subcutaneously in a volume of 0.1 ml./20 g. mouse and all other drugs were administered intraperitoneally in a volume of 0.2 ml./20 g. mouse. In any individual experiment, the total volume of fluid injected was kept constant by using aliquots of 0.9% NaCl as substitute for a drug.

The organophosphates used were: (1) organophosphates with a diethylphosphoryl group: ethyl pyrophosphate (TEPP), diethyl *p*-nitrophenylphosphate (E 600), 3-(diethoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3-0340), 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3-0422), and (2) organophosphates with a di*iso*propyl phosphoryl group: dyflos or di*iso*propyl phosphorofluoridate (DFP), di*iso*propyl *p*-nitrophenylphosphate (D 600), 3-(di*iso*propoxyphosphinyloxy)-pyridine (Ro 3-0351).

The organophosphates will be referred to throughout the text by the abbreviations given in brackets. TEPP, DFP, and Ro 3-0351 were made up as 10% w/v and E 600 as 1% w/v stock solutions in ethanol, and 0.9% NaCl was used for all subsequent dilutions. Ro 3-0340 and Ro 3-0422 were always dissolved freshly in 0.9% NaCl. The poor water-solubility of D 600 made it necessary to use ethanolic solutions at all dose levels.

Atropine was used as the sulphate and references in the text apply to the salt.

For pyridine-2-aldoxime methiodide the abbreviation P-2-AM is used in preference to P_2AM (Hobbiger, 1956).

In vitro *Reactivation by P-2-AM.*—Since TEPP, E 600, Ro 3-0422 and DFP are hydrolysed enzymatically by liver and other tissues, the following technique was used to reduce interference by free organophosphates with the reactivation process (Hobbiger, 1955 and 1956). A group of several mice was injected with 1 mg./kg. atropine to avoid changes in haemoconcentration resulting from excessive fluid loss following cholinesterase inhibition. Thirty minutes later a sublethal dose of an organophosphate was injected, and after a further interval of not less than 30 min. blood was collected by cardiac puncture under ether anaesthesia. The individual blood samples were heparinized and pooled. The brains were then removed and homogenized in 10 parts of 0.025 M $NaHCO_3$. The effect of different concentrations of P-2-AM on the inhibited enzymes was investigated in the Warburg apparatus under the same conditions as used for the estimation of cholinesterase activity. 0.3 ml. of a solution containing 2 vol. blood or brain homogenate and 1 vol. P-2-AM (dissolved in 0.025 M $NaHCO_3$) were added to the side arm of each manometric vessel and tipped into the main compartment after various periods of incubation; the latter contained 2.7 ml. of substrate dissolved in 0.025 M $NaHCO_3$. Cholinesterase activity was recorded over three consecutive periods of 20 min. The activities of P-2-AM treated blood or brain and of controls for inhibition and normal enzyme activity were determined concurrently and % reactivation was calculated from the expression: 100 × (activity of reactivated enzyme − activity of inhibited enzyme):(activity of control enzyme obtained from untreated mice − activity of inhibited enzyme).

In vivo *Effect of P-2-AM on Phosphorylated True Cholinesterases.*—All mice were injected with 1 mg./kg. atropine. One group served as controls for normal cholinesterase activity, another group was injected with a sublethal dose of an organophosphate 30 min. after the atropine had been given, and the remaining mice had in addition to the organophosphate an injection of 25 mg./kg. P-2-AM either 5 min. before or 30 min. after the organophosphate. Two mice of each group were sacrificed at various intervals and cholinesterase activities of whole blood and brain in each group were determined on pooled samples. Blood was collected by the technique described previously and brains were homogenized for 2 min. in 40 parts of 0.025 M $NaHCO_3$ immediately after their removal from the body. 0.3 ml. of blood and 1 ml. of brain homogenate respectively were then added without delay to the main compartment of each manometric vessel, and the total volume of fluid was adjusted to 2.7 ml. by addition of 0.025 M $NaHCO_3$. The manometric vessels were at once transferred to the Warburg bath and gassed for 5 min. with a mixture of 95% N_2+5% CO_2. After allowing for temperature equilibration, the substrate (0.3 ml.) was added to the enzyme from a sidearm and enzyme activity was recorded over 3 consecutive periods of 15 min.

Experiments in which the activity of aliquots of the same blood sample was determined after different periods of incubation in the Warburg bath showed that the 1:9 dilution of the blood which was used in these experiments was not always sufficient to arrest *in vitro* reactivation of diethylphosphoryl cholinesterase. This applied particularly to blood samples taken at 30 or 35 min. after the injection of P-2-AM and to a smaller extent to blood samples taken 1 hr. after the injection of P-2-AM (Fig. 1). To

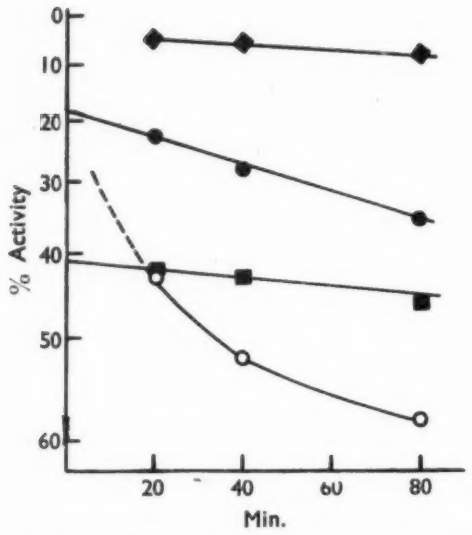

FIG. 1.—*In vitro* reactivation of phosphorylated true cholinesterases in blood samples taken from mice which have been injected with P-2-AM and an organophosphate. The mice received either 25 mg./kg. P-2-AM 5 min. before the injection of 0.4 mg./kg. TEPP (●, ○, ■) or TEPP alone (◆). 0.3 ml. blood was used in each Warburg vessel and incubated either together with 8 parts 0.025 M $NaHCO_3$ in the main compartment (●, ■, ◆) or without dilution in the sidearm (○). ●, ○, ◆: blood collected 30 min. after the injection of TEPP; ■: blood collected 60 min. after the injection of TEPP. The intercepts with the ordinate are taken as the true enzyme activity at the time when the blood was collected. Abscissa: min. of incubation before addition of substrate to the enzyme (●, ■, ◆) or enzyme to substrate (○). Ordinate: activity as % of controls (logarithmic scale).

minimize the error resulting from *in vitro* reactivation the activities of aliquots of each blood sample were determined after different periods of incubation in the Warburg bath; the activities of these blood samples were then used to obtain the true enzyme activity by extrapolation, as shown in Fig. 1. The activities of individual blood samples shown in Fig. 3 and Table I were all obtained by this procedure.

No *in vitro* reactivation was observed when brain homogenates were used.

In vivo *Protection against Organophosphate Poisoning.*—The lethal dose of each organophosphate was determined in otherwise untreated mice and in mice which were injected with 25 mg./kg. P-2-AM, 50 mg./kg. atropine, and 25 mg./kg. P-2-AM+50 mg./kg. atropine. With each individual organophosphate all observations were made concurrently on the same batch of mice. Atropine was injected 30 min. before and P-2-AM 5 min. before the organophosphate; the same time interval was adhered to in mice which received both drugs. The amounts of organophosphate which were injected were arranged in a geometric progression. In each group, 4 mice received the same treatment and a period of 6 hr. was used as the criterion of survival. By this technique only twofold changes in the lethal dose of an organophosphate or multiples of it could be recorded.

Measurement of Cholinesterase Activity.—Cholinesterase activity was measured by the manometric technique at 37° in a 0.025 M $NaHCO_3$ solution and in an atmosphere of 95% N_2+5% CO_2. The hydrolysis of 0.03 M (±)-acetyl-β-methylcholine chloride was used as a measure of the activity of the true cholinesterases in blood and brain.

Mouse blood, unlike human blood, contains true cholinesterase in both the red cells and the plasma. In a pooled sample of blood which was obtained from 6 mice and which had a packed cell volume of 45%, the red cells contributed 71% and plasma 29% towards the hydrolysis of (±)-acetyl-β-methylcholine. Experiments in which P-2-AM was used failed to disclose any difference in behaviour between the phosphorylated true cholinesterases of red cells and plasma, and thus it seems likely that they are either identical or closely related enzymes.

RESULTS

In vitro *Reactivation of Phosphorylated True Cholinesterases in Mouse Blood and Brain by P-2-AM*

True Cholinesterases in Blood.—Thirty minutes after the subcutaneous injection of 50 to 75% of the lethal dose of an organophosphate the activity of the true cholinesterases in blood was reduced by at least 75%. If blood samples were collected at this time and incubated with suitable concentrations of P-2-AM, more than 80% of the activity of the inhibited enzymes was restored by the oxime. Under the experimental conditions (see Methods) identical rates of reactivation were obtained by a fixed concentration of P-2-AM if inhibition was produced by organophosphates which have a common dialkylphosphoryl group. Within the observed range, namely up to 80% reactivation, reactivation proceeded at a rate characteristic for a first order reaction (Fig. 2).

It has been shown previously that the rate of interaction between P-2-AM and the organophosphates used in this work is determined by the organophosphates themselves and not their dialkylphosphoryl group (Hobbiger, 1956). The results with P-2-AM thus indicate that the inhibition of true cholinesterases in mouse blood by organophosphates is the result of enzyme phosphorylation, and the inhibited enzymes will, therefore, be referred to in the text as phosphorylated true cholinesterases or phosphorylated enzymes.

The concentrations of P-2-AM which were required under the experimental conditions to restore 50% of the activity of the phosphorylated true cholinesterases in mouse blood within 1 hr were 4.2×10^{-6} M for the diethylphosphoryl

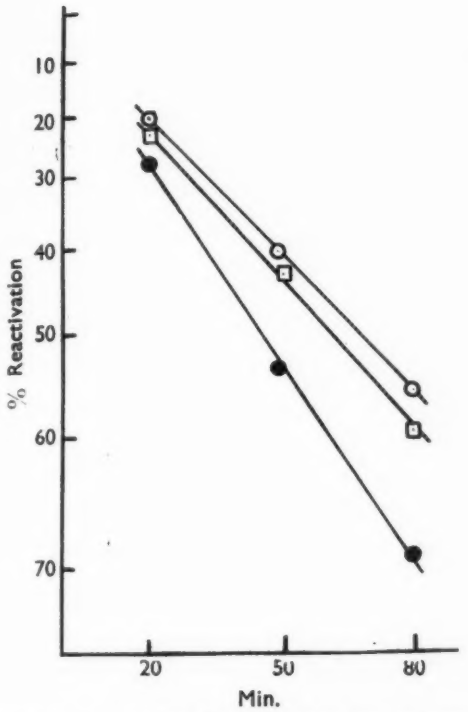

FIG. 2.—*In vitro* reactivation of phosphorylated true cholinesterases in blood. Blood was collected 30 min. after the injection of 0.4 mg./kg. E 600 and 2.5 mg./kg. DFP respectively. The true cholinesterases in blood of the mice injected with E 600 were inhibited to an extent of 91% and for reactivation 7.5×10^{-6} M P-2-AM (●—●) and 2.5×10^{-6} M P-2-AM (○—○) were used. The inhibition in the DFP injected mice amounted to 95% and for reactivation 1×10^{-4} M P-2-AM (□——□) was used. Abscissa: duration of reactivation. Ordinate: % reactivation of inhibited (phosphorylated) enzyme (logarithmic scale).

enzymes and 1.4×10^{-1} M for the di*iso*propylphosphoryl enzymes.

If reactivation was carried out in a medium which contained 0.075 M NaCl, 0.075 M KCl, 0.04 M $MgCl_2$ and 0.1% bovine plasma albumin in addition to 0.025 M $NaHCO_3$, such as in the medium used in earlier work with phosphorylated human true cholinesterase (Hobbiger, 1956), higher concentrations of P-2-AM were required to achieve an effect equal to that obtained with 0.025 M $NaHCO_3$ as medium. Experiments in which a mixture consisting of equal parts of phosphorylated true cholinesterases of the mouse and human was incubated with P-2-AM failed to show any difference in the sensitivity to P-2-AM between corresponding phosphorylated enzymes obtained from the two species. The reduction in the effectiveness of P-2-AM when used in a medium of 0.025 M $NaHCO_3$, 0.075 M NaCl, 0.075 M KCl, 0.04 M $MgCl_2$ and 0.1% bovine plasma albumin was mainly due to the presence of $MgCl_2$ (unpublished results).

True Cholinesterase in Brain.—P-2-AM also reactivated the phosphorylated true cholinesterase in brain homogenates (obtained from the brains of mice which had been injected with TEPP, E 600, DFP or D 600), and the results obtained in these experiments are consistent with the interpretation that inhibition is the result of enzyme phosphorylation. However, to achieve equal rates of reactivation of corresponding phosphorylated enzymes in blood and brain the concentration of P-2-AM had to be approximately doubled if the latter were used. This does not necessarily mean that the phosphorylated true cholinesterases in blood and brain have different sensitivities to P-2-AM, since crude enzyme preparations were used and a variety of factors, such as differences in *p*H, salts, proteins, etc., could easily account for the observed discrepancies.

Reactivation After Prolonged Inhibition.—The inhibition of human, bovine and rabbit true cholinesterases by organophosphates involves the following stages: cholinesterase + organophosphate→addition complex→phosphorylated cholinesterase which can be reactivated by nucleophilic reagents→phosphorylated cholinesterase which cannot be reactivated by nucleophilic reagents (Hobbiger, 1956; Davies and Green, 1956).

The same sequence of reactions also applies to the phosphorylation of the true cholinesterases of the mouse. If P-2-AM was added to mouse blood which had been collected at different intervals after the injection of an organophosphate it was found that the effectiveness of P-2-AM was inversely related to the duration of enzyme inhibition. The phosphorylated true cholinesterase in brain behaved like the corresponding enzymes in blood. The rate of formation *in vivo* of a phosphorylated true cholinesterase which cannot be reactivated by P-2-AM showed the characteristics of a first order reaction and was determined by the dialkylphosphoryl group of the organophosphate. The time required to convert 50% of the phosphorylated enzyme which can be reactivated by P-2-AM into a phosphorylated enzyme which cannot be reactivated by P-2-AM was approximately 36 hr. for the diethylphosphoryl enzyme and 4 hr. for the di*iso*propylphosphoryl enzyme.

In vivo *Effect of P-2-AM on the Phosphorylated True Cholinesterases of Mouse Blood and Brain*

Experiments with Organophosphates which Form a Diethylphosphoryl Enzyme.—The activity of the true cholinesterases in blood of atropinized

mice was reduced by at least 75% within 30 min. after the injection of 0.4 mg./kg. TEPP or E 600, 5 mg./kg. Ro 3-0340 or 0.02 mg./kg. Ro 3-0422. Spontaneous reactivation of the phosphorylated true cholinesterase *in vivo* was slow, and only a small increase in enzyme activity took place during a period of 2 hr. following the injection of the organophosphate.

Mice which were injected with 25 mg./kg. P-2-AM either 5 min. before or 30 min. after the administration of TEPP, E 600, Ro 3-0340, or Ro 3-0422 showed consistently higher activities of the true cholinesterases in blood than the mice of a control group which were injected with the organophosphate alone. P-2-AM was most effective when given at a time when inhibition had reached a maximum and the higher enzyme activities in P-2-AM treated mice could only be the result of a reactivation process. Differences in the degree of reactivation between groups of mice injected with TEPP, E 600, Ro 3-0340 or Ro 3-0422 were small and of an order similar to those observed in different experiments with the same organophosphate. Fig. 3 shows a plot of the reactivation in an experiment with TEPP and Table I gives the range over which individual results are spread.

If mice were injected with P-2-AM and an organophosphate all blood samples taken within 1 hr. after the injection of the oxime showed reactivation *in vitro* on incubation at 37° in an atmosphere of 95% N_2 + 5% CO_2; this reactivation *in vitro* was greatest in undiluted blood samples and under the experimental conditions always incomplete (Fig. 1).

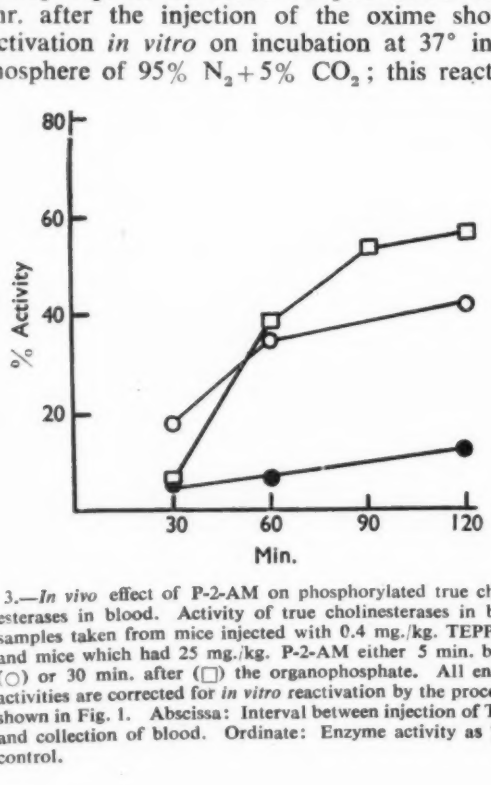

FIG. 3.—*In vivo* effect of P-2-AM on phosphorylated true cholinesterases in blood. Activity of true cholinesterases in blood samples taken from mice injected with 0.4 mg./kg. TEPP (●) and mice which had 25 mg./kg. P-2-AM either 5 min. before (○) or 30 min. after (□) the organophosphate. All enzyme activities are corrected for *in vitro* reactivation by the procedure shown in Fig. 1. Abscissa: Interval between injection of TEPP and collection of blood. Ordinate: Enzyme activity as % of control.

TABLE I

IN VIVO EFFECT OF P-2-AM ON THE PHOSPHORYLATED TRUE CHOLINESTERASES IN BLOOD OF MICE INJECTED WITH A SUBLETHAL DOSE OF TEPP, E 600, Ro 3-0340 OR Ro 3-0422

The degree of reactivation was calculated as follows: % reactivation of phosphorylated enzyme $= 100 \times \frac{a-b}{c-b}$ where a, b, and c represent the activities of the true cholinesterases in blood samples. a, refers to mice injected with P-2-AM and an organophosphate; b, to mice injected with an organophosphate alone; and c, to mice used as controls. All enzyme activities are corrected for *in vitro* reactivation (see Methods and Fig. 1).

Min. after Injection of P-2-AM	% Reactivation if 25 mg./kg. P-2-AM is Injected	
	5 Min. Before Organophosphate	30 Min. After Organophosphate
30–35	10–24	31–39
60–65	27–35	45–52
120–125	33–40	49–56

The true cholinesterase of brain was only inhibited in those mice which were injected with 0.4 mg./kg. TEPP or E 600; 0.02 mg./kg. Ro 3-0422 or 5 mg./kg. Ro 3-0340 had no effect on it. Experiments in which 25 mg./kg. P-2-AM was injected either 5 min. before or 30 min. after 0.4 mg./kg. TEPP or E 600 was given showed that the oxime had only a small effect on the phosphorylated cholinesterase of the brain (Table II) and the highest degree of reactivation obtained in any individual experiment was 7%. Control experiments in which the brains of mice which had been injected with either E 600 or E 600 and P-2-AM were homogenized together with the brains taken from normal mice showed that no measurable inhibition took place during homogenization and incubation in the Warburg bath; thus it is unlikely that *in vitro* artifacts obscured any reactivation which had taken place *in vivo*.

TABLE II

EFFECT OF 25 MG./KG. P-2-AM ON THE ACTIVITY OF TRUE CHOLINESTERASE IN BRAIN OF MICE INJECTED WITH 0·4 MG./KG. E 600

	Activity as % of Controls After	
	3 Hr.	6 Hr.
Experiment 1:		
E 600 only	14	17
P-2-AM 5 min. before E 600 ..	16	23
P-2-AM 30 min. after E 600 ..	15	16
Experiment 2:		
E 600 only	16	16
P-2-AM 5 min. before E 600 ..	14	20
P-2-AM 30 min. after E 600 ..	18	19

Experiments with Organophosphates which Form a Diisopropylphosphoryl Enzyme.—An

injection of 25 mg./kg. P-2-AM had no consistent effect on the phosphorylated true cholinesterases of blood or brain if the mice were injected with DFP, D 600 or Ro 3-0351.

Protection against Lethal Organophosphate Poisoning by P-2-AM, Atropine and a Combination of Atropine and P-2-AM

If mice were injected with 25 mg./kg. P-2-AM 5 min. before the administration of TEPP, E 600, Ro 3-0422 or Ro 3-0340 different degrees of protection were obtained against lethal doses of these organophosphates. Table III shows that the protection is greatest in those mice which were

TABLE III

PROTECTION AGAINST LETHAL ORGANOPHOSPHATE POISONING

Column a gives the lethal dose for mice in mg./kg. Protection is shown in columns b, c, and d and expressed in multiples of the lethal dose (given in column a) required to cause death under the experimental conditions. Whenever a range is given partial survival was obtained with the lower dose and all mice died with the higher dose. Columns b, c, and d refer to the following conditions: b, 25 mg./kg. P-2-AM was given 5 min. before the organophosphate. c, 50 mg./kg. atropine was given 30 min. before the organophosphate. d, 50 mg./kg. atropine and 25 mg./kg. P-2-AM were given 30 min. and 5 min. respectively before the organophosphate.

Organophosphate	Lethal Dose in mg./kg. (a)	Multiples of Lethal Dose		
		(b)	(c)	(d)
TEPP	0·7	2	1–2	32
Ro 3-0422 ..	0·04	2	1–2	8–16
E 600	0·6	2–4	2	>128
Ro 3-0340 ..	10	8	1–2	32–64
DFP	5	1–2	2	16–32
Ro 3-0351 ..	25	1–2	1–2	8

injected with Ro 3-0340, less in the mice which were injected with E 600, and smallest if TEPP or Ro 3-0422 were used.

The protection by P-2-AM against lethal doses of organophosphates which form a di*iso*propylphosphoryl enzyme was far smaller than that against organophosphates which form a diethylphosphoryl enzyme. A dose of 25 mg./kg. P-2-AM saved the life of less than 50% of the mice which were injected with one lethal dose of DFP or Ro 3-0351 and no protection was observed in experiments with D 600.

If P-2-AM treated mice were injected with a dose of an organophosphate which was lethal under these conditions all the characteristic sequelae of cholinesterase inhibition, like salivation, diarrhoea and overactivity of striped muscle, were seen. This suggested that the protection by P-2-AM against lethal doses of organophosphates might be enhanced by atropine.

Mice which were injected with 50 mg./kg. atropine 30 min. before a lethal dose of the organophosphate was given showed only nicotinic but no muscarinic symptoms of acetylcholine poisoning. The antidotal effect of 50 mg./kg. atropine was smaller than that of 25 mg./kg. P-2-AM if TEPP, E 600, Ro 3-0340 or Ro 3-0422 were used, but for DFP, D 600 and Ro 3-0351 the reverse was true (Table III).

Mice which were injected with 50 mg./kg. atropine and 25 mg./kg. P-2-AM were protected to a greater extent than would have been expected from an additive effect of the two antidotes, and the degree of protection was determined by the organophosphate itself and not by its dialkylphosphoryl group (Table III). The majority of the mice died within 20 min. of the injection of a dose of an organophosphate which was lethal under these conditions, and all mice which survived the first hour remained alive during the period of observation of 6 hr. The findings with E 600 and DFP were in agreement with the results obtained by Kewitz *et al.* (1956).

No further protection was obtained if, in addition to atropine and P-2-AM, 100 mg./kg. nikethamide, 7 mg./kg. picrotoxin, or 60 mg./kg. leptazol were injected intraperitoneally together with the oxime.

Mice which were injected with 25 mg./kg. l-hyoscine and 25 mg./kg. P-2-AM showed the same degree of protection against TEPP as those mice which received 50 mg./kg. atropine and 25 mg./kg. P-2-AM. On the other hand, the combination of hyoscine and P-2-AM was approximately twice as effective as the combination of atropine and P-2-AM in Ro 3-0422 poisoning.

Effect of Ethanol.—The results obtained with D 600 are not included in Table III, since its low solubility in water necessitated the use of ethanolic solutions with all doses and ethanol itself modified the results of toxicity tests to a considerable extent.

Mice which had been injected subcutaneously with 0.1 ml. ethanol became less lively, swayed and walked unsteadily when roused; this effect lasted from 2 to 4 hr. If ethanolic solutions of the organophosphates were injected, the lethal dose was approximately double that found when aqueous solutions of the organophosphates were used; this also applied to mice which had been pretreated with either P-2-AM or atropine. Mice which had received both atropine and P-2-AM and were then injected with higher doses of ethanolic solutions of the organophosphates became unconscious within a short time after the injection of the organophosphate and showed a considerable reduction in rate and depth of respiration. Under these conditions it became exceedingly difficult to establish the exact time of

death, especially since the incidence of death was spread over the entire period of observation (6 hr.) and the mice just " faded away " without terminal convulsions or other noticeable symptoms. Experiments in which TEPP and Ro 3-0340 were injected into mice which had received both

TABLE IV

EFFECT OF ETHANOL ON THE PROTECTION AGAINST LETHAL ORGANOPHOSPHATE POISONING

For explanation of cols. a, b, c, and d, see Table III.

Organophosphate	Lethal Dose in mg./kg. (a)	Multiples of Lethal Dose		
		(b)	(c)	(d)
TEPP :				
Aqueous ..	0·7	2	1–2	32
Ethanolic ..	1·4	2	2	8
Ro 3-0340 :				
Aqueous ..	10	8	1–2	32–64
Ethanolic ..	20	2	1	16

atropine and P-2-AM showed that the protection obtained under these conditions was less than that observed when aqueous solutions of the organophosphates were used (Table IV).

DISCUSSION

The interaction between true cholinesterase and an organophosphate leads to the formation of a phosphorylated enzyme which is enzymically inactive. Thus the injection of an organophosphate is followed by an accumulation of acetylcholine (which is constantly released from its store at various sites) and corresponding symptoms (Douglas and Paton, 1951 ; Stewart, 1952 ; Barnes and Duff, 1954). Acute death from organophosphate poisoning always appears to be the result of respiratory failure, although its character varies from one species to another (Modell and Krop, 1946 ; Candole, Douglas, Evans, Holmes, Spencer, Torrance, and Wilson, 1953). If acetylcholine is indeed responsible for the acute lethal effects of organophosphate poisoning one would expect that antagonists of its actions should be useful antidotes. During the past 20 years a number of competitive antagonists of the muscarinic actions of acetylcholine have been tested in this respect and found to give only a limited degree of protection, even when used in high doses. This also applies to antagonists of the nicotinic actions of acetylcholine. On the other hand, animals which receive positive pressure ventilation in addition to atropine (Barnes, 1953) or which are pretreated with antagonists of the muscarinic as well as the nicotinic actions of acetylcholine show a far greater degree of protection (Candole and McPhail, 1954 ; Parkes and Sacra, 1954 ; Lewis, McKeon, and Lands, 1955). This seems to indicate that it is not the accumulation of acetylcholine at any particular site which is responsible for death in acute organophosphate poisoning, but that some mechanism is involved which requires the accumulation of acetylcholine at various sites and which is also linked to respiration.

The danger connected with the combined use of several antidotes, each of which might produce lethal effects on its own, necessitates an entirely new approach, for example reactivation of phosphorylated cholinesterases or enzyme protection, for the treatment of organophosphate poisoning.

It has recently been found that relatively small amounts of certain oximes speedily restore the activity of freshly phosphorylated true cholinesterases *in vitro* (Childs *et al.*, 1955 ; Wilson and Ginsburg, 1955). The results presented in this paper show that in the mouse pyridine-2-aldoxime methiodide (P-2-AM), which was the most active oxime found so far, was less effective in reactivating phosphorylated true cholinesterases *in vivo* than was indicated by the *in vitro* experiments. According to the findings of Askew, Davies, Green, and Holmes (1956) with related oximes one would expect that, after an intraperitoneal injection of 25 mg./kg. P-2-AM, a concentration of 1 to 4×10^{-4} M of the oxime would be maintained in the blood for at least 15 min. In *in vitro* experiments, such concentrations were sufficient to restore within a few minutes more than 90% of the activity of true cholinesterases if they were inhibited by organophosphates which formed a diethylphosphoryl enzyme. However, the *in vivo* reactivation during 30 min. amounted to only 10 to 24% if 25 mg./kg. P-2-AM was injected 5 min. before TEPP, E 600, Ro 3-0340, or Ro 3-0422, and varied from 31 to 39% if the oxime was injected 30 min. after the organophosphate. Since the oxime was given intraperitoneally in a volume of 0.2 ml./20 g. mouse, it was possible that the rate of absorption was unfavourable and the concentrations of P-2-AM in blood have to be measured before the *in vivo* results can be fully correlated with the *in vitro* results. Davies and Green (1956) have shown that the active factor in the reactivation process is the anion of the oxime, and differences in *p*H could also be partly responsible for the observed differences between *in vitro* and *in vivo* effectiveness of P-2-AM. The finding that blood samples which were collected 30 min. after the injection of TEPP or other organophosphates in P-2-AM treated mice contained an effective

concentration of the oxime but could only be partly reactivated during incubation at 37° in the Warburg cannot be interpreted at the present moment, since nothing is known about protein binding and the metabolism of P-2-AM under the experimental conditions; accelerated transphosphorylation (Hobbiger, 1956; Davies and Green, 1956) has also to be considered in this connexion.

The time course of reactivation of phosphorylated true cholinesterases indicates that the concentration of the oxime in blood falls below an effective level within 1 to 2 hr. after a single injection of 25 mg./kg. P-2-AM. This is in agreement with the findings of Kewitz *et al.* (1956), who studied the protection against E 600 in mice which had received 40 mg./kg. P-2-AM intraperitoneally at various intervals before the injection of the organophosphate and found that the antidotal effect of the oxime was greatly reduced within 2 hr.

A dose of 25 mg./kg. P-2-AM had only a negligible effect on the phosphorylated true cholinesterases in the brain of mice injected with TEPP or E 600, although the oxime was a highly effective reactivator when allowed to act on brain homogenates. P-2-AM behaved in this respect like other quaternary ammonium compounds, such as organophosphates (Burgen and Hobbiger, 1951; Hobbiger, 1954; Koelle and Steiner, 1956). Mayer and Bain (1956) have shown that the chief barrier to the entrance of quaternary ammonium compounds into the brain lies between the outer wall of the capillary endothelium and the glial plasma membrane and that this barrier can be overcome by higher doses. Thus the failure of P-2-AM to reach the brain is not absolute, and with higher doses of the oxime a greater degree of reactivation has been obtained (Kewitz and Nachmansohn, 1957).

25 mg./kg. P-2-AM was without any *in vivo* effect if the true cholinesterases were inhibited by organophosphates which formed a di*iso*propylphosphoryl enzyme (DFP, Ro 3-0351 or D 600). This was to be expected from the effect of P-2-AM on the inhibition produced by organophosphates which form a diethylphosphoryl enzyme since the *in vitro* experiments showed that the di*iso*propylphosphoryl enzyme was approximately 30 times less sensitive to P-2-AM than the diethylphosphoryl enzyme.

If one compares the *in vivo* effect of P-2-AM on phosphorylated true cholinesterases with its antidotal effect in lethal organophosphate poisoning it is difficult to interpret the latter entirely in terms of cholinesterase reactivation as has been postulated by Kewitz and Wilson (1956), Kewitz *et al.* (1956), Kewitz (1957) and Kewitz and Nachmansohn (1957). Progressive enzyme reactivation took place during a period of 1 to 2 hr. following the injection of the organophosphate if mice were injected with 25 mg./kg. P-2-AM 5 min. before a sublethal dose of an organophosphate which forms a diethylphosphoryl enzyme (TEPP, E 600, Ro 3-0340 or Ro 3-0422). However, both *in vitro* and *in vivo* experiments failed to show that P-2-AM was more effective in restoring enzyme activity if inhibition was produced by Ro 3-0340 or E 600 than if inhibition was produced by TEPP or Ro 3-0422. It is thus difficult to understand why protection by P-2-AM decreased in the order: Ro 3-0340 > E 600 > TEPP ≡ Ro 3-0422. Different degrees of protection were also found if the mice received atropine as well as P-2-AM before the injection of the organophosphate. E 600 and Ro 3-0340 are far more stable than TEPP or Ro 3-0422, and one is tempted to speculate that differences in the rates of *in vivo* phosphorylation of true cholinesterases account for the observed differences in protection. Experiments with organophosphates which form a di*iso*propylphosphoryl enzyme (DFP and Ro 3-0351) speak against such an interpretation. Mice which were pretreated with atropine and P-2-AM were protected to a greater extent against DFP than against Ro 3-0422, and protection against TEPP was only slightly greater than protection against DFP. Since P-2-AM had no measurable effect on the inhibition produced by DFP it is difficult to see how the protection obtained by the combined use of atropine and P-2-AM could be entirely the result of enzyme reactivation. Askew (1956), who studied the antidotal properties of a series of oximes, was also unable to find any direct correlation between their potency as reactivators of sarin-inhibited cholinesterase *in vitro* and their effectiveness as antidotes of sarin poisoning of rats *in vivo*.

It is possible that the actions of P-2-AM are not restricted to phosphorylated cholinesterases and that other actions play some part in the protection against organophosphates under certain conditions, for example, when mice are injected with atropine and P-2-AM. Holmes and Robins (1955) have shown that P-2-AM blocks neuromuscular transmission in the isolated phrenic nerve diaphragm of the rat in concentrations which are required for enzyme reactivation. Furthermore, P-2-AM is an anticholinesterase in its own right (Hobbiger, 1956). Both neuromuscular blocking

agents and combinations of atropine and eserine are antidotes of lethal organophosphate poisoning. The protection obtained by the combined use of atropine and eserine is particularly effective, although eserine on its own is only a very feeble antidote (Koster, 1946 ; DuBois, Doull, Okinaka and Coon, 1953). Thus eserine shares with P-2-AM the property of greatly potentiating the effect of atropine.

It is widely believed that organophosphates possess other actions in addition to those mediated by cholinesterase inhibition (for references, see Murtha, McNamara, Edberg, Bergner, and Wills, 1955 ; Cohen and Posthumus, 1955 ; Groblewski, McNamara, and Wills, 1956 ; Naess, 1956). Perhaps some of the differences observed in the experiments reported here are caused by them.

Askew (1956) has recently shown that the antidotal potency of a number of oximes varies between different species. This has to be borne in mind before the results in the mouse, especially the antidotal effects obtained by the combined use of atropine and P-2-AM, are generalized.

The present work shows that the results obtained with one particular organophosphate (Kewitz *et al.*, 1956 ; Askew, 1956) are not applicable to other organophosphates which form the same type of phosphorylated enzyme. Screening of new oximes for their antidotal properties should, therefore, include as many organophosphates as possible and by doing so organophosphate insecticides could be selected which are less dangerous than some of those which are widely used nowadays.

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References

Aldridge, W. N., and Davison, A. N. (1953). *Biochem. J.*, **55**, 763.
Askew, B. M. (1956). *Brit. J. Pharmacol.*, **11**, 417.
—— Davies, D. R., Green, A. L., and Holmes, R. (1956). Ibid., **11**, 424.
Barnes, J. M. (1953). Ibid., **8**, 208.
—— and Duff, J. I. (1954). Ibid., **9**, 153.
Burgen, A. S. V., and Hobbiger, F. (1951). Ibid., **6**, 593.
Candole, C. A. de, Douglas, W. W., Evans, C. L., Holmes, R., Spencer, K. E. V., Torrance, R. W., and Wilson, K. M. (1953). Ibid., **8**, 466.
—— and McPhail, M. K. (1954). *Nature, Lond.*, **174**, 552.
Childs, A. F., Davies, D. R., Green, A. L., and Rutland, J. P. (1955). *Brit. J. Pharmacol.*, **10**, 462.
Cohen, J. A., and Posthumus, C. H. (1955). *Acta physiol. pharmacol. neerl.*, **4**, 17.
Davies, D. R., and Green, A. L. (1956). *Biochem. J.*, **63**, 529.
Douglas, W. W., and Paton, W. D. M. (1951). *J. Physiol.*, **115**, 71P.
DuBois, K. P., Doull, J., Okinaka, A. J., and Coon, J. M. (1953). *J. Pharmacol.*. **107**, 464.
Groblewski, G. E., McNamara, B. P., and Wills, J. H. (1956). Ibid., **118**, 116.
Hobbiger, F. (1954). *Brit. J. Pharmacol.*, **9**, 159.
—— (1955). Ibid., **10**, 356.
—— (1956). Ibid., **11**, 295.
Holmes, R., and Robins, E. L. (1955). Ibid., **10**, 490.
Kewitz, H. (1957). *Arch. Biochem. Biophys.*, **66**, 263.
—— and Nachmansohn, D. (1957). Ibid., **66**, 271.
—— and Wilson, I. B. (1956). Ibid., **60**, 261.
—— —— and Nachmansohn, D. (1956). Ibid., **64**, 456.
Koelle, G. B., and Steiner, E. C. (1956). *J. Pharmacol.*, **118**, 420.
Koster, J. B. (1946). Ibid., **88**, 39.
Lewis, J. R., McKeon, Jr., W.B., and Lands, A. M. (1955). *Arch. int. pharmacodyn.*, **102**, 371.
Mayer, St. E., and Bain, J. A. (1956). *J. Pharmacol.*, **118**, 17.
Modell, W., and Krop, S. (1946). Ibid., **88**, 34.
Murtha, E. F., McNamara, B. P., Edberg, L. J., Bergner, A. D., and Wills, J. H. (1955). Ibid., **115**, 291.
Naess, K. (1956). *Acta pharm. tox., Kbh.*, **12**, 154.
Parkes, M. W., and Sacra, P. (1954). *Brit. J. Pharmacol.*, **9**, 299.
Stewart, W. C. (1952). Ibid., **7**, 270.
Wilson, I. B., and Ginsburg, S. (1955). *Arch. Biochem. Biophys.*, **18**, 168.

Brit. J. Pharmacol. (1957), **12**, 447.

PHARMACOLOGICAL PROPERTIES OF ESTERS OF 1-ALKYL-2-HYDROXYALKYLPYRROLIDINE AND THEIR QUATERNARY DERIVATIVES

BY

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A series of esters of 1-alkyl-2-hydroxyalkylpyrrolidine and their quaternary derivatives have been shown to possess significant anti-acetylcholine activity. The benzilic acid esters were the most active, followed by xanthene-9-carboxylic acid, fluorene-9-carboxylic acid and diphenylacetic acid esters in that order. The quaternary derivatives were more active than their corresponding tertiary compounds both *in vivo* and *in vitro*. The most active compound of the series tested *in vivo* was (1-methylpyrrolid-2-yl)methyl benzilate methiodide and was as potent as atropine. There was a progressive decrease in anti-acetylcholine activity and a proportional increase in local anaesthetic activity as the number of carbon atoms was increased from 1 to 3 in the pyrrolidyl side-chain of the tertiary salts of the benzilic acid ester series. Likewise increasing the size of the group on the nitrogen atom led to a decrease in anti-acetylcholine activity and an increase in local anaesthetic activity. Quaternization of the tertiary salts resulted in a loss of local anaesthetic activity. Most of the compounds tested possessed some antihistamine properties, while papaverine-like activity was confined to the tertiary salts only. No significant neuromuscular blocking activity was evident.

There have been numerous attempts to modify the structure of atropine with a view to minimizing its undesirable side effects, and many simple esters have been prepared which have shown anti-acetylcholine activity. This paper describes the pharmacology of the esters of pyrrolidyl alcohols synthesized by Doyle, Mehta, and Sach (unpublished observations). Of these compounds only the benzilic and diphenylacetic acid esters of 1-methyl-2-hydroxymethylpyrrolidine have previously been reported (Blicke and Lu, 1955).

METHODS

Tests in Vitro

Anti-acetylcholine Action. — Segments, approximately 2 cm. long, were removed from the lower ileum of young guinea-pigs weighing not more than 250 g. and suspended in oxygenated Tyrode solution at 37° in a 5 ml. bath. Acetylcholine (ACh), which was allowed to act for 30 sec., was added at 2 min. intervals until constant responses were obtained. The doses of atropine and test compound were added 30 sec. before ACh and were adjusted so that the ACh response was reduced to approximately 30% and 70% of the original. Between the successive doses of antagonist the ACh response was allowed to return to normal. A 16-point assay was then performed using a Latin square design (Fisher and Yates, 1953) and the results analysed by the procedure of Schild (1942).

Segments 2 to 3 cm. long, cut from the mid-portion of the jejunum of young rabbits weighing approximately 1.5 kg., were suspended in Tyrode solution at 37°. An identical statistical procedure to that used for the guinea-pig ileum anti-ACh assay was employed. A 4 min. cycle was used. The antagonist was added 60 sec. before the ACh. Fresh rabbit jejunum often gave irregular responses to ACh for the first 1 or 2 hr.; regular responses were obtained more rapidly, without loss in sensitivity or accuracy, with gut which had been stored for 18 hr. at 7°.

Antihistamine Action.—Preparations of guinea-pig ileum were made as for the anti-acetylcholine test. Histamine was added at 90 sec. intervals until the responses were regular. The antagonist was then added to the bath 30 sec. before the histamine and the depression in responses compared with that obtained by a standard antihistamine drug. If the activity of the unknown exceeded that of the standard, a 16-point Latin square design assay was performed.

Papaverine-like Activity.—A strip of rabbit jejunum 2 to 3 cm. long was suspended in Tyrode solu-

tion at 37° in a 5 ml. bath. Spasm was induced with barium chloride (250 μg./ml.) and the spasmolytic added 2 min. later, while the barium chloride was still present. The compound was allowed to act for a further 2 min. and then washed out. The degree of relaxation of the barium spasm produced by the compound under test was compared with that produced by papaverine HCl. Two further washings were performed at 5 min. intervals during the 15 min. cycle employed.

Curare-like Activity.—The rat phrenic nerve-diaphragm preparation, as described by Bülbring (1946), was used.

Langendorff Heart.—The heart from a guinea-pig weighing approximately 400 g. was removed under ether anaesthesia and connected by an aortic cannula to a perfusion system. Oxygenated Locke solution containing $NaHCO_3$ 0.5 g./l. was allowed to flow through a warming coil to the heart. The perfusion pressure was maintained between 60 and 70 cm. water. Drugs were injected in 0.05 to 0.1 ml. Locke solution close to the heart. The amplitude and frequency were recorded and the rate of coronary outflow was measured by a Gaddum drop recorder.

Tests in Vivo

Acute Toxicity.—Acute intravenous toxicities were estimated in male albino mice (18 to 22 g.); 10 mice were used with each dose and the results were analysed according to the method described by Finney (1952).

Anti-acetylcholine Action on Cat Blood Pressure.—The method of Kühl (1925) was used. Cats were anaesthetized with ether followed by chloralose-urethane mixture (40 mg./kg. chloralose, 160 mg./kg. urethane intravenously). Blood pressure recordings were made from the carotid artery and injections were made through a polythene cannula inserted into the femoral vein. The compounds were dissolved in saline.

A dose of ACh (0.5 to 1.0 μg.), which gave a just submaximal depression in blood pressure, was administered intravenously at 2 min. intervals until a regular depression of the blood pressure was obtained. The % depression of the ACh response caused by a small intravenous dose of spasmolytic given 1 min. previously was measured and matched with that caused by a dose of atropine (0.2 to 5.0 μg.). Small doses were employed so that recovery was rapid and several compounds could be assayed on each animal. Each drug was tested on several animals and the results averaged.

Intestinal Motility.—Decerebrate cats were used. A midline stab incision was made in the abdomen. The intestine was drawn out through the incision and glass cannulae were inserted into the duodenum and jejunum or ileum. The cannulae were bent at right angles so that when in position the longer arm was vertical. A ligature was tied round the intestine 5 to 10 cm. from the cannula and the loop was filled with liquid paraffin to give an internal pressure of about 10 cm. water. The cannula was connected to an air tambour and the intestine replaced in the abdominal cavity. Handling of the intestine was reduced to a minimum. After 2 to 3 hr., when the effects of the anaesthetic had worn off, kymographic recordings of intestinal movements were made. Compounds were injected intravenously in saline through a cannula inserted in the femoral vein.

Anti-acetylcholine Activity on Guinea-pig Colon.—Guinea-pigs were anaesthetized with urethane (2 g./kg. subcutaneously), and a cannula was tied into the colon. Carbachol (1 to 2 μg./guinea-pig) was injected intravenously, at 3 to 5 min. intervals, through a cannula in the jugular vein until the intestine responded regularly by contraction. A single dose of compound was administered intravenously and two further injections of carbachol were made. If there was no response to these, the drug was considered to be active at the dose given. From 2 to 6 animals were used at each dose level. The activity was expressed in terms of minimal effective dose.

Mydriatic Activity.—Assays for mydriatic activity were carried out on the mouse according to the method of Pulewka as used by Ing, Dawes, and Wajda (1945). The pupil diameter was measured in arbitrary units with the aid of a micrometer scale set in the eyepiece of a dissecting microscope (×10). Compounds were given subcutaneously or orally to groups of 5 mice (18 to 22 g.). The pupil diameter was measured at 5 min. intervals over a period of 30 min. Atropine was used as a standard. The results were assessed at the time of maximum activity, which was usually 20 min. after administration.

Local Anaesthetic Activity.—The intradermal weal method described by Bülbring and Wajda (1945) and modified by Somers and Edge (1947) was used to determine local anaesthetic activity. Guinea-pigs weighing 400 to 450 g. were employed.

A modification of the corneal reflex method described by Chance and Lobstein (1944) was also used. Three dose levels of both test and standard compound were employed and 2 guinea-pigs were used for each dose. The solutions of the compounds were made in phosphate buffer at *p*H 7.3. The standard (procaine HCl) solution was applied to one eye and the test solution to the other. The cornea was then touched lightly with a thin bristle 6 times every 2 min. for 20 min., and the number of times that the animal failed to blink was noted. After a 4 hr. period, the same animals were used again in a crossover test. The results, expressed as % response, were plotted against log concentration and analysed according to the method described by Burn, Finney, and Goodwin (1950).

Salivary Flow.—The effect of compounds on salivary flow was estimated by the method employed by Issekutz (1917), as modified by Brown and Quinton (1957).

Gastric Secretion.—The method used was essentially that of Shav, Komarov, and Fels (1945). Six male albino rats weighing 100 to 200 g. were starved for 18 to 24 hr. in cages with wide-mesh wire bottoms to

prevent coprophagy. The duodenum was then ligated under ether anaesthesia and 4 ml. of warm saline was given intraperitoneally. Three rats were given 5 mg./kg. of the test compound subcutaneously, while the remaining three were given 1.0 ml. saline. The animals were returned to their cages and killed 6 hr. later. The oesophagus was ligated and the stomach was removed and cleared of adhering mesentery, dried with blotting paper, and the contents drained through a slit into the centrifuge tube. The gastric contents were centrifuged for 10 min. at approximately 4,000 rev./min. and the total volume and the volume of solid contents measured. 1 ml. of the supernatant was titrated with N/100 NaOH for free and total acid, Topfer's reagent being used as indicator.

Neuromuscular Blocking Action.—The decerebrate cat gastrocnemius-sciatic nerve preparation was used. Stimuli of 2 to 3 V., frequency 7/min., were applied to the sciatic nerve. Compounds at 1/20th and 1/10th of their respective intravenous LD50 values were administered in saline at 10 min. intervals through a cannula in the femoral vein.

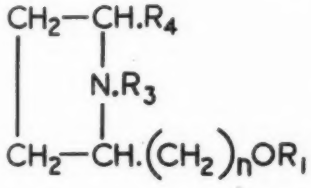
Results

Anti-acetylcholine Activity on Isolated Guinea-pig Ileum and Rabbit Jejunum.—The result of the anti-ACh activity of each compound on the isolated guinea-pig ileum (Table I) represents the weighted mean of at least four separate assays. The standard error of the combined mean values was calculated, giving on average a standard error of the order of 5% ($P=0.05$). At least two anti-ACh assays were also performed on the rabbit jejunum, and, as a rule, the relative potencies were less, generally by a factor of about 25%, than

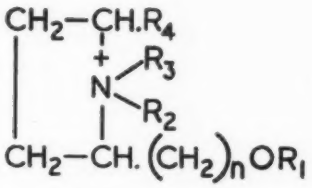
TABLE I

INTRAVENOUS TOXICITIES, ANTI-ACETYLCHOLINE AND SPASMOLYTIC ACTIVITIES OF A SERIES OF TERTIARY AND QUATERNARY SALTS OF PYRROLIDYL ALCOHOLS ESTERIFIED WITH VARIOUS ACIDS. ACID RADICAL R_1 DERIVED FROM DIPHENYLACETIC ACID (GROUP A); BENZILIC ACID (GROUP B); XANTHENE-9-CARBOXYLIC ACID (GROUP C); FLUORENE-9-CARBOXYLIC ACID (GROUP D)

The asterisk indicates an ester of the secondary base.



Tertiary esters



Quaternary esters

Ester	BRL No.	n	R_2	R_3	R_4	LD50 (intraven.) mg./kg.	Anti-ACh: Isol. G-Pig Ileum	Anti-ACh: Cat B.P.	Mydriasis	Saliv. Flow	Anti-Hist. (Antazoline SO_4=1·0)	Anti-$BaCl_2$ Spasm. (Papaverine HCl=1·0)
							(Atropine=1·0)					
						Tertiary Esters						
A	373	2	—	CH_3	H	26·0	0·58	0·01	—	—	0·08	1·0
A	432	2	—	C_2H_5	H	15·0	0·22	0·01	—	—	0·04	1·0
B	485	1	—	CH_3	H	32·5	0·65	0·10	0·35	0·02	1·0	1·0
*B	539	2	—	H	H	27·0	0·61	0·01	<0·05	0·04	0·02	0·4
B	416	2	—	CH_3	H	28·5	0·99	0·10	0·19	0·06	0·13	0·75
B	433	2	—	C_2H_5	H	15·4	0·64	0·1	0·13	0·05	0·02	0·8
B	529	2	—	C_3H_7	H	10·5	0·18	0	<0·05	0·01	1·0	2·0
B	447	2	—	CH_3	CH_3	15·5	0·36	0·01	<0·05	0·01	0·15	1·0
B	490	3	—	CH_3	H	11·5	0·12	0	0·1	0	0·01	1·0
C	448	2	—	CH_3	H	24·5	0·93	0·01	—	—	0·15	1·0
D	417	2	—	CH_3	H	21·5	0·48	0·01	—	—	0·20	1·5
						Quaternary Esters						
A	424	2	CH_3	CH_3	H	3·0	0·45	0·03	—	—	0·1	0
B	499	1	CH_3	CH_3	H	21·5	1·02	1·0	1·09	2·03	0·08	0
B	526	1	CH_3	C_2H_5	H	14·0	1·29	0·5	1·03	0·42	0·06	0
B	425	2	CH_3	CH_3	H	22·0	2·02	0·15	0·17	0·69	0·1	0
B	481	2	CH_3	C_2H_5	H	13·0	2·72	0·40	1·33	0·95	0·15	0
B	494	2	C_2H_5	C_2H_5	H	10·0	0·23	0·06	0·25	0·06	0·03	0
B	521	2	CH_3	C_3H_7	H	8·9	0·24	0·12	0·28	0·19	0·01	0
B	454	2	CH_3	CH_3	CH_3	12·0	0·09	0	<0·05	0·01	0·01	0
B	564	3	CH_3	CH_3	H	5·4	0·53	0	<0·15	0·02	0	0
C	453	2	CH_3	CH_3	H	4·7	1·18	0·2	—	—	0·01	0
D	438	2	CH_3	CH_3	H	4·4	0·45	0·07	—	—	—	0
Atropine						82·0	1·00	1·0	1·0	1·0	0·03	0
Atropine methonitrate						8·8	0·87	1·5	—	1·65	0	0
Homatropine						84·0	0·26	—	—	0·01	0·01	0
Oxyphenonium						16·0	0·64	0·4	0·93	0·62	0	0
Methantheline						6·6	1·73	0·25	0·12	—	0·01	0
Propantheline						3·9	2·09	1·0	0·6	1·13	0·15	0

those obtained on the guinea-pig ileum. The most active compound tested on isolated guinea-pig ileum was β-(1-methylpyrrolid-2-yl)ethyl benzilate methiodide (BRL 481). On the other hand (1-methylpyrrolid-2-yl)methyl benzilate (BRL 485) was the most active compound of the series on the rabbit jejunum. On this tissue, however, methantheline was the most active of all the compounds tested. The quaternary derivatives were more active than their corresponding tertiary salts.

Antihistamine, Papaverine-like, and Curare-like Actions in Vitro.—Most of the compounds showed some detectable antihistamine action, compounds BRL 485 and BRL 529 having activities equal to that of antazoline sulphate. Most of the tertiary salts exhibited a papaverine-like action against barium-chloride-induced spasms of the rabbit jejunum equal to that of papaverine HCl. Compound BRL 529 was the most potent, being twice as active as papaverine. Quaternization destroyed or greatly reduced this property. Potencies less than 0.05 of papaverine HCl were taken as nil.

No significant curare-like action could be detected in any of the compounds.

Isolated Mammalian Heart.—Atropine in single doses up to 100 μg. usually had no effect on the amplitude, or was slightly stimulant; it had no effect on the rate or coronary flow of the perfused heart. In doses over 20 μg. the tertiary amine esters of benzilic acid appeared to have negative inotropic and chronotropic actions, which were particularly marked with BRL 416. A dose of 50 μg. of this compound usually produced a pronounced decrease in all cardiac functions leading to complete ventricular arrest. The secondary base BRL 539 and the ethyl-compound BRL 433 were equally depressant, whereas the short- and long-chain homologues BRL 485 and BRL 490 showed less activity in this respect. The quaternary amines had generally no action or a slight positive inotropic effect with high doses.

Toxicities.—The acute intravenous toxicities of the compounds are given in Table I. In general there was an increase in toxicity with increase in length of the side chain attached to the pyrrolidine nucleus and with the size of the group attached to the nitrogen atom. Quaternization of the tertiary compounds invariably led to greater toxicity, although the tertiary salts showed signs of possessing central stimulant properties absent from the quaternary derivatives.

BRL 485 and BRL 416 were the least toxic of the tertiary salts, while BRL 499 and BRL 425, their methyl quaternary analogues, were approximately one and a half times as toxic, but were the least toxic of the quaternary salts. On the other hand, atropine methonitrate was much more toxic than atropine.

Anti-acetylcholine Effect on Blood Pressure.—The results obtained are given in Table I. Compound BRL 499 was the most active of the compounds tested in the BRL series and was equal to propantheline in this respect. Compounds BRL 526 and BRL 481 had also considerable activity. The recovery from most of the compounds followed much the same pattern as after atropine.

Gastro-intestinal Motility.—There was no apparent relationship between the effect of drugs on the gastro-intestinal motility of the cat and their action in antagonizing acetylcholine on the blood pressure of the cat. An inhibitory response of the intestine was usually obtained from the first injection of the drug, but the response to a second injection was variable. In many experiments a second response was frequently not obtained and the gut became completely refractory to anti-ACh drugs. This made the interpretation of results and a direct comparison of activities difficult. We also found that the charcoal meal test in mice gave no clear guide to intestinal inhibition by anti-ACh drugs.

Anti-acetylcholine Action on Guinea-pig Colon.—In view of the wide variability obtained with the preceding method and the uncertainty of correct interpretation, it was decided to use carbachol as an intestinal stimulant, and to note the depressant effect of compounds on this response. Difficulties were again experienced in that the intestines of some animals were found to be completely resistant to carbachol, even though profuse salivation occurred in response to the injections. Furthermore, a response to carbachol tended to appear and disappear spontaneously during the course of the experiment. For this reason the experiments were designed to be of short duration, and all animals which failed to respond to an initial dose

TABLE II

MINIMUM EFFECTIVE INTRAVENOUS DOSE REQUIRED TO INHIBIT CARBACHOL-INDUCED CONTRACTION OF GUINEA-PIG COLON

Compound	Minimum Effective Dose (μg./kg.)
Atropine	8
Oxyphenonium ..	32
Propantheline ..	Variable 16 to 64
BRL 485	Variable
BRL 499	8
BRL 526	64
BRL 416	64
BRL 425	16
BRL 433	64
BRL 481	32
BRL 521	64

of carbachol were discarded. The results obtained with the more active compounds of the benzilic acid series (group B) are given in Table II. A typical response to carbachol and its inhibition by an anti-ACh drug (BRL 499) is seen in Fig. 1. This compound proved to be the most active synthetic compound tested.

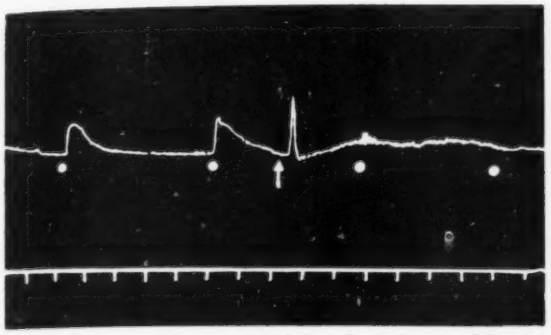


FIG. 1.—Recording of the response of the guinea-pig colon following 1 μg. carbachol administered intravenously at the dots and the inhibition produced by compound BRL 499 (16 μg. kg.) given at the arrow. Time, 1 min.

Mydriasis.—All the benzilic acid esters were tested for their mydriatic action. By the subcutaneous route compounds BRL 481, BRL 526 and BRL 499 were all of the same order of activity as atropine. Oxyphenonium and propantheline were somewhat less active and all the other members of the BRL series were much less active.

Given orally, only the tertiary salts showed regular absorption and gave repeatable results, but none was as active as atropine. The ratio of potencies of BRL 485 and BRL 416 to atropine between the subcutaneous and oral routes was, however, of the same order. The quaternary salts were absorbed erratically and required a dose of the order of 100 times the subcutaneous dose to show an effect. Even with such a dose, no significant absorption occurred in a considerable % of animals.

Local Anaesthetic Activity.—Selected compounds were tested for local anaesthetic activity, which was found in tertiary salts but not in quaternary salts. The results are given in Table III.

Salivary Flow.—The regression for inhibition of salivation by atropine against log dose was linear in two sets of 8 rabbits. Dose-response curves were also found for compounds BRL 416 and BRL 425 on the first set of rabbits and for compound BRL 499, oxyphenonium, propantheline and atropine methonitrate on the second set of rabbits. Since none of the curves obtained showed

TABLE III

COMPARISON OF LOCAL ANAESTHETIC ACTIVITY OF TERTIARY SALTS WITH PROCAINE HCl

The effect of increasing the number of carbon atoms in side chain, the size of group on the nitrogen atom, and of altering the acidic radical is recorded. See Table I for explanation of n, R_3, and R_4.

Compound	n	R_3	R_4	Corneal Reflex (Procaine HCl = 1)	Intradermal Weal (Procaine HCl = 1)
BRL 485 ..	1	CH_3	H	2·05	0·2 to 0·5
BRL 416 ..	2	CH_3	H	16·5	2·9
BRL 490 ..	3	CH_3	H	29·3	4·9
BRL 433 ..	2	C_2H_5	H	26·6	8·6
BRL 447 ..	2	CH_3	CH_3	28·6	
BRL 529 ..	2	C_9H_7	H	36·5	7·4
BRL 539 ..	2	H	H	0·2 to 0·5	
BRL 373 ..	2	CH_3	H	13·8	

significant deviation from parallelism or linear regression, it was decided to estimate activity at one dose level and to assume that the dose-response line passing through the point obtained would run parallel to the standard atropine curve. The dose which gave a 50% depression in response (ED50) was found by interpolating on the line so obtained. The relative potencies are given in Table I.

Gastric Secretion.—A quantitative assay was impractical owing to the large variation in the amount of acid secreted in untreated rats. The activities have, therefore, been measured in terms of an " all or none " response. All the compounds tested produced a significant reduction ($P=0.05$) in the volume of gastric secretion, except compounds BRL 485, BRL 447, BRL 454, BRL 481, and BRL 417.

Cat Gastrocnemius-sciatic Preparation.—None of the compounds had any significant action on the response of the gastrocnemius muscle to sciatic nerve stimulation.

DISCUSSION

All the compounds of the BRL series showed anti-ACh activity both *in vitro* and *in vivo*. On the basis of the *in vitro* comparative assays, the benzilic acid esters were the most active, followed by xanthene-9-carboxylic acid, fluorene-9-carboxylic acid and diphenylacetic acid esters, in that order.

Detailed study of the benzilic acid esters showed that maximum activity on the guinea-pig ileum occurred when the carbon side-chain attached to the pyrrolidine nucleus contained two carbon atoms. When the side-chain was kept constant, an increase in the size of the group on the nitrogen atom resulted in a proportional decrease in *in vitro* anti-ACh activity.

There was little difference between the tertiary compounds containing one or two carbon atoms in the side-chain in their antagonism of the vaso-depressor action of ACh, although the shorter chain compound appeared to be more active as a mydriatic and the two carbon chain compound more active as an anti-salivatory agent. The three carbon chain compound was, however, much less active as an anti-ACh agent. The short chain compound tended to be somewhat stimulatory and produced variable results against carbachol stimulation of the guinea-pig colon. The precise activity could not be measured, but appeared to be much less than that shown by the two carbon chain compound. The three carbon compound had little activity.

A feature of the tertiary derivatives was their local anaesthetic activity, the quaternary compounds being completely inactive in this respect. As the anti-ACh action decreased, the local anaesthetic activity increased in proportion to the number of carbon atoms in the side-chain or attached to the nitrogen. Relative to procaine hydrochloride the compounds were much more active on the corneal reflex test than by the intradermal weal test, indicating that they penetrate mucous membranes. On the corneal reflex β-(1-ethylpyrrolid-2-yl)ethyl benzilate (BRL 433), however, appeared to be more active than β-(1-*n*-propylpyrrolid-2-yl)ethyl benzilate (BRL 529), which has a larger group on the nitrogen. This discrepancy is probably due to the error of the assay, which, by the nature of the test, was large.

All the tertiary salts were active orally. This was well shown by the oral mydriatic test. In mice, absorption from the alimentary tract was poor and erratic with all quaternary derivatives. Tests in human volunteers, however, indicated that BRL 499 was regularly absorbed at a dose of 5 mg. after oral administration.

The quaternary compounds having the same groups on the nitrogen atom showed maximum anti-ACh activity *in vitro* and *in vivo* when the side-chain contained only one carbon atom. (1-methyl-2-pyrrolid-2-yl)methyl benzilate methiodide (BRL 499) was the most active of all the compounds tested *in vivo*, and in every test was as potent as atropine. When the side-chain of the quaternary compounds was kept constant and the alkyl groups on the nitrogen atom increased in size, maximum *in vitro* activity occurred with the methyl-ethyl-derivatives (BRL 481 and BRL 526). In the two carbon chain series the corresponding methyl-propyl-compound (BRL 521) was comparatively much less active and had the same order of activity as the diethyl-compound (BRL 494). *In vivo*, the relative potencies of this series on salivary flow and mydriasis were greater than those for their action against ACh on the cat blood pressure and against carbachol-induced stimulation of the guinea-pig colon. In this latter test, however, activity was maximal with the dimethyl-compound (BRL 425).

The substitution of a methyl group in the 5-position in the pyrrolidine ring greatly reduced anti-ACh activity, but did not appear to influence local anaesthetic activity. Anti-ACh activity appears to depend, therefore, on the overall size of the group or groups on the nitrogen atom, on the length of the side-chain, and on the absence of other substituents in the pyrrolidine nucleus.

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References

Blicke, F. F., and Lu, C. J. (1955). *J. Amer. chem. Soc.*, **77**, 29.
Brown, D. M., and Quinton, R. M. (1957). *Brit. J. Pharmacol.*, **12**, 63.
Bülbring, E. (1946). Ibid., **1**, 38.
—— and Wajda, I. (1945). *J. Pharmacol.*, **85**, 78.
Burn, J. H., Finney, D. J., and Goodwin, L. G. (1950). *Biological Standardisation*. London: Oxford University Press.
Chance, M. R. A., and Lobstein, H. (1944). *J. Pharmacol.*, **82**, 203.
Finney, D. J. (1952). *Probit Analysis*. London: Cambridge University Press.
Fisher, R. A., and Yates, F. (1953). *Statistical Tables for Agricultural, Biological and Medical Research*, 4th ed., p. 70. Edinburgh: Oliver and Boyd.
Ing, H. R., Dawes, G. S., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 85.
Issekutz, B. (1917). *Zeitschr. f. Exp. Path. u. Therap.*, **19**, 99.
Kühl, G. (1925). *Arch. exp. Path. Pharmak.*, **109**, 295.
Schild, H. O. (1942). *J. Physiol.*, **101**, 115.
Shay, H., Komarov, S. A., and Fels, S. S. (1945). *Gastroenterology*, **5**, 43.
Somers, G. F., and Edge, N. D. (1947). *Quart. J. Pharm. Pharmacol.*, **20**, 380.

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ACTION OF ANAPHYLACTIC SHOCK AND ANAPHYLATOXIN ON MAST CELLS AND HISTAMINE IN RATS

BY

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(RECEIVED MAY 17, 1957)

Anaphylactic shock in rats produces disruption of mast cells. These cells are also disrupted when rat mesentery is incubated with antigen *in vitro*. The plasma histamine reaches a maximum about 5 min. after injection of the antigen. Though antihistamines protect rats against anaphylactic shock, they do not prevent mast cell disruption. Previous depletion by compound 48/80 of the histamine bound to the mast cells prevents anaphylactic shock and the increase in plasma histamine. Anaphylatoxin produces no mast cell alterations or plasma histamine increase, and thus does not seem to act as a histamine-releasing agent in rats. Probably nearly all the histamine liberated in anaphylaxis in rats comes from the mast cells.

It is well known that histamine is released from tissues in anaphylaxis. It has recently been shown that most of the tissue histamine is located in the mast cells (Riley and West, 1953; Riley, 1953a), whence it can be displaced by histamine-releasing substances (Riley, 1953b; Mota, Beraldo, and Junqueira, 1953; Mota, Junqueira, Beraldo, and Ferri, 1954; Mota, Beraldo, Ferri, and Junqueira, 1956).

There has been considerable dispute as to whether true anaphylaxis occurs in rats (Dragstedt, 1941). But Halpern, Liacopoulos, and Castillo (1955) have recently shown that it can be regularly induced in rats with bovine serum albumin as antigen, and I have produced it consistently with alum-precipitated horse serum as antigen.

The present experiments were undertaken to study the action of a specific antigen on mast cells and histamine in rats. Since anaphylatoxin is a strong histamine-releasing agent (Rocha e Silva, Bier, and Aronson, 1951), and is able to duplicate the main features of anaphylactic shock in guinea-pigs, its effect on the histamine and mast cells was also investigated in rats.

METHODS

Wistar rats of both sexes weighing 300 to 400 g. were used. The antigen was filtered horse serum stored under aseptic conditions. Immediately before use the serum was precipitated by adding an equal amount of a 2.5% solution of potassium alum. The animals were sensitized with 1 ml. of precipitated serum subcutaneously, and 1 ml. of non-precipitated serum intraperitoneally, given daily for three days. The animals were challenged 18 to 30 days later with 1 ml./100 g. of body weight of horse serum intravenously. As controls, non-sensitized rats were given the same volume of horse serum.

Rat serum anaphylatoxin was prepared as described by Rothschild and Rocha e Silva (1954), and injected intravenously (1 ml./100 g. body weight). The activity of anaphylatoxin was tested by its effect on the isolated guinea-pig ileum.

For microscopical observation of the mast cells, the skin of the snout, the lips, the ears, and the tongue were fixed by injecting fixative through the carotid artery. The mesentery was fixed by immersion, stained, and examined in whole-mount preparations. The fixative was 50% aqueous ethanol containing 10% formaldehyde and 5% acetic acid. Fixation was allowed to proceed overnight, and frozen sections, 50 μ thick, were stained with toluidine blue.

Histamine assays were performed on the atropinized guinea-pig ileum according to Feldberg and Talesnik (1953). All histamine values are given as base.

RESULTS

Effect of Anaphylactic Shock and Anaphylatoxin on Mast Cells.—All 10 sensitized rats injected with the antigen gave reactions 3 min. after the injection, the most common being weakness, prostration, and difficulty in breathing causing retraction of the lower ribs. Scratching was noted in some animals. Most of these signs subsided within 1 hr. and all the animals had recovered

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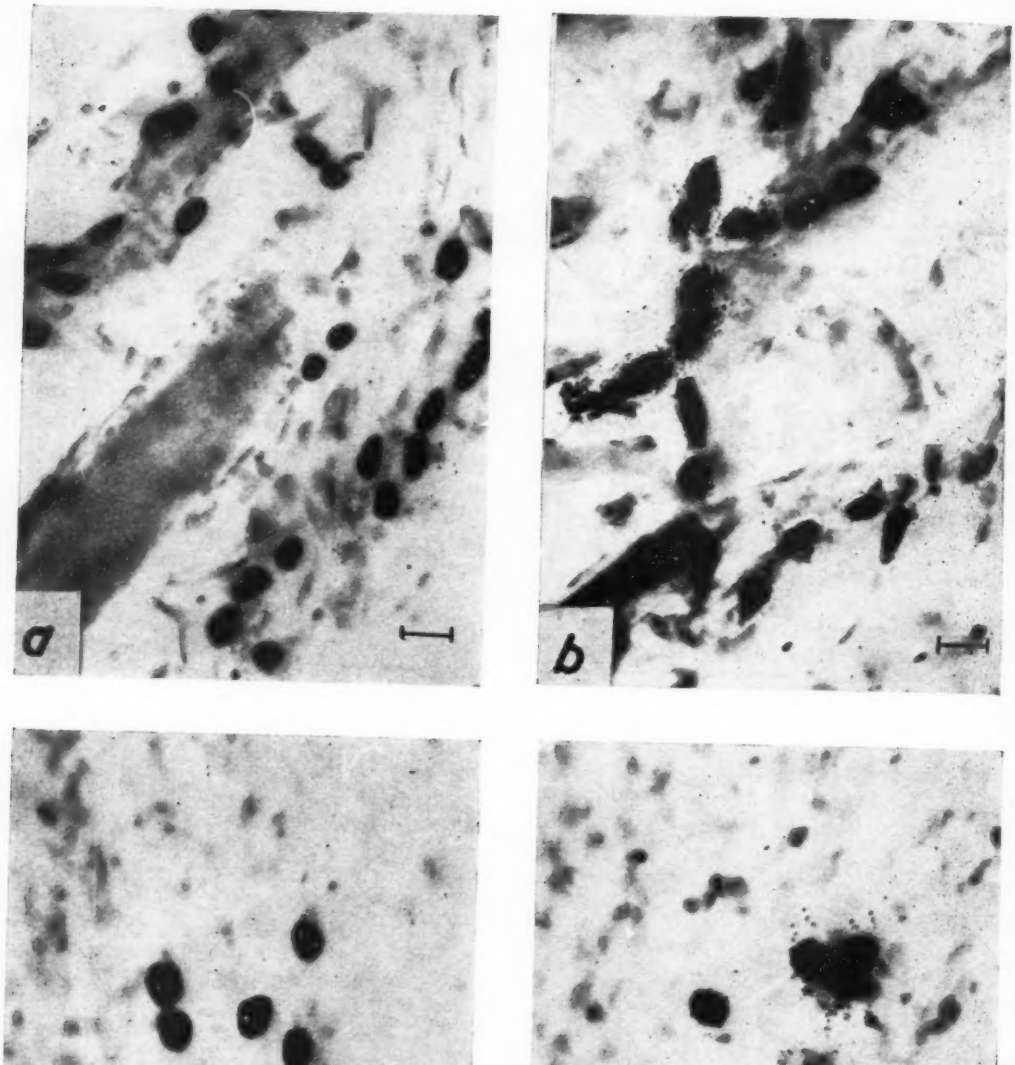

FIG. 1.—(*a*) Skin of a non-sensitized rat injected with horse serum showing normal mast cells. (*b*) Skin of a sensitized rat injected with horse serum showing disrupted mast cells. (*c*) Mesentery of a non-sensitized rat incubated with horse serum showing normal mast cells. (*d*) Mesentery of a sensitized rat incubated with horse serum showing disrupted mast cells. Scale in all photomicrographs = 20 μ.

3 hr. later when they were killed with ether. Although some mast cells kept their usual morphology and the number of altered mast cells varied from animal to animal, microscopical examination of the tissues showed constant and definite alterations of these cells, represented by granule extrusion, very similar to those prompted by the injection of compound 48/80 (Mota *et al.*, 1953). These alterations, present only in the shocked rats, were most conspicuous in the skin (Fig. 1*b*), less so in the tongue, and frequently absent from the mesentery.

None of the 20 rats injected with anaphylatoxin reacted. All of them were killed with ether 3 hr. after the injection, and on microscopical examination no mast cell alterations were observed.

Since adrenalectomy increases the number of reactive mast cells under conditions in which histamine liberation occurs (unpublished observation), anaphylatoxin was also injected to adrenalectomized rats: no mast cell alterations were detected.

In vitro *Experiments.*—Since mast cells of the mesentery react *in vitro* with histamine liberators (Mota *et al.*, 1953; Norton, 1954), similar experiments were conducted with antigen and with anaphylatoxin.

Sensitized rats were anaesthetized with ether and bled from the jugular vein. Pieces of mesentery were carefully dissected out and placed in cold isotonic sucrose. Two or three pieces were then placed in horse serum or rat serum anaphylatoxin for 15 min. at 37°, removed, fixed, stained, and examined. As a control, pieces of mesentery from non-sensitized rats were incubated in the same way with horse serum or non-activated rat serum. Microscopical observation showed mast cell disruption in the mesentery of the sensitized rats (Fig. 1*d*); but no alterations were seen in the mast cells of the non-sensitized controls. In the experiments with anaphylatoxin, in spite of the direct contact of this agent with the tissue, there were no alterations in the mast cells. However, if guinea-pig mesentery is incubated with rat serum anaphylatoxin very conspicuous alterations of the mast cells occur which are similar to those induced *in vitro* by the antigen in tissues of sensitized guinea-pigs.

Effect on Plasma Histamine.—Since blood histamine is increased in guinea-pigs and dogs subjected to anaphylactic shock (Code, 1939), the free histamine level of the plasma of shocked rats was determined.

The rats were lightly anaesthetized by injecting intravenously 2 ml./kg. of a mixture containing 10% of pentobarbitone sodium and 20% of urethane. Blood samples (1 to 2 ml.) were collected by puncture of the jugular vein in a heparin-containing syringe, before, and 3, 5, 10, 15, and 20 min. after the injection of the antigen or anaphylatoxin, and the plasma immediately obtained by centrifugation at 0° and assayed for histamine.

The results of these experiments are shown in Table I. Administration of the specific antigen to sensitized animals led to high plasma histamine levels. The maximum was reached in about 5 min., and then declined so that no histamine was detectable at 20 min. No increase in plasma histamine was detected in rats given anaphylatoxin.

TABLE I

PLASMA HISTAMINE CONCENTRATIONS (μG./ML.) IN SENSITIZED RATS BEFORE AND AFTER INJECTION OF THE SPECIFIC ANTIGEN

Rat	Before Injection	Time After Injection (min.)				
		3	5	10	15	20
1	0·0	0·10	1·50	0·00	0·00	0·0
2	0·0	0·15	0·10	0·10	0·00	0·0
3	0·0	1·00	1·20	1·20	0·05	0·0
4	0·0	0·15	0·05	0·05	0·00	0·0
5	0·0	1·00	1·00	0·50	0·15	0·0
6	0·0	1·00	1·20	0·50	0·10	0·0

Effect of Antihistamines.—In order to see the importance of histamine in the production of anaphylactic shock in rats, the protective effect of antihistamines was tried. From a group of 20 sensitized rats, 10 received intraperitoneally 5 mg. of chlorprophenpyridamine maleate (Alergon), and 1 hr. later all the rats were given antigen. Although all the non-protected rats had severe reactions, the rats pre-treated with antihistamine showed only mild shock. Since adrenalectomy makes rats particularly sensitive to anaphylaxis (Flashman, 1926; Wyman, 1929) these experiments were repeated with adrenalectomized animals. In a group of 10 sensitized and adrenalectomized rats given antigen, 8 died; in a similar group protected with the antihistamine, all survived. However, microscopical examination showed the same mast cell alterations in both groups.

Effect of Previous Treatment with Compound 48/80.—Since repeated injections of compound 48/80 deplete the histamine bound to the mast cells of rats (Mota *et al.*, 1956), it was decided to verify the effect of pre-treatment with compound 48/80 on anaphylactic shock. Thus, from a group of 20 sensitized rats, 10 were given daily intraperitoneal injections of each of the following

doses of compound 48/80: 0.1, 0.1, 0.2, 0.3, 0.4, 0.5, 0.5, 1.0 mg., while 10 others were given corresponding volumes of saline. Twenty-four hours after the last injection, both groups of animals were given antigen. The reactions in the animals treated with 48/80 were minimal, whereas those in the control rats were severe. Furthermore, in another group of 5 rats treated with 48/80, no plasma histamine could be detected after the injection of the antigen.

Discussion

Mast cell alterations in anaphylactic shock were referred to in dogs by Jaques and Waters (1941), and described in guinea-pigs by Mota and Vugman (1956). The modifications presented by these cells in anaphylaxis in rats are more conspicuous and easier to detect than those in guinea-pigs under the same conditions. However, under ordinary conditions of sensitization only in about one-third of the sensitized rats could definite mast cell alterations be found (Mota, 1953). It is interesting to note that only some mast cells present alterations ; the others maintain their usual morphology. We have also observed this after giving small doses of 48/80 to rats. Perhaps these responsive cells represent the source of the mobilizable histamine. It cannot be said whether the anaphylactic reaction takes place in the mast cells themselves, or whether the alterations of the mast cells are a consequence of a reaction taking place elsewhere. However, the hypothesis is tempting that the mast cell might contain antibodies, possibly adsorbed on its surface—which would account for histamine release occurring only when the antigen is applied to the intact cell, as shown by Copenhaver, Nagler, and Goth (1953) and Mongar and Schild (1956). Further experimental work is needed on this point.

It is known that in rats most of the tissue histamine is located in the mast cells or in unknown cells of the wall of the digestive tract (Mota *et al.*, 1956). Thus, both could be the source of the histamine liberated in anaphylactic shock in this species. Rats injected repeatedly with compound 48/80, and thus depleted of histamine bound to the mast cells, are protected from anaphylactic shock, and show no plasma histamine increase ; this strongly suggests that histamine liberated during anaphylactic shock originates in the mast cells.

It has been shown that 5-hydroxytryptamine is a natural constituent of rat mast cells (Benditt, Wong, Arase, and Roeper, 1955), and that substances, such as compound 48/80, that disrupt mast cells (Mota *et al.*, 1953) release 5-hydroxytryptamine from the tissues (Bhattacharya and Lewis, 1956). It is therefore probable that, in anaphylaxis in rats, 5-hydroxytryptamine and other substances are released besides histamine. However, no activity was observed in the plasma of the shocked rats that could not be inhibited by antihistamines. Furthermore, the fact that antihistamines protect rats against anaphylactic shock suggests that histamine is responsible for the major part of the syndrome of anaphylactic shock in this species.

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References

Benditt, E. P., Wong, R. L., Arase, M., and Roeper, E. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 303.
Bhattacharya, B. K., and Lewis, G. P. (1956). *Brit. J. Pharmacol.*, **11**, 202.
Code, C. F. (1939). *Amer. J. Physiol.*, **127**, 78.
Copenhaver, J. H., Nagler, M. E., and Goth, A. (1953). *J. Pharmacol.*, **109**, 401.
Dragstedt, C. A. (1941). *Physiol. Rev.*, **21**, 563.
Feldberg, W., and Talesnik, J. (1953). *J. Physiol.*, **120**, 550.
Flashman, D. H. (1926). *J. infect. Dis.*, **38**, 461.
Halpern, B. N., Liacopoulos, P., and Castillo, C. P. (1955). *C.R. Soc. Biol., Paris*, **149**, 314.
Jaques, L. B., and Waters, E. T. (1941). *J. Physiol.*, **99**, 454.
Mongar, J. L., and Schild, H. O. (1956). Ibid., **131**, 207.
Mota, I. (1953). M.D. Thesis, University of São Paulo, Brazil.
—— Beraldo, W. T., and Junqueira, L. C. U. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 455.
—— —— Ferri, A. G., and Junqueira, L. C. U. (1956). *Ciba Foundation Symposium on Histamine* (p. 47). London: Churchill.
—— Junqueira, L. C. U., Beraldo, W. T., and Ferri, A. G. (1954). *Nature, Lond.*, **173**, 547.
—— and Vugman, I. (1956). Ibid., **177**, 427.
Norton, S. (1954). *Brit. J. Pharmacol.*, **9**, 494.
Riley, J. F. (1953a). *J. Path. Bact.*, **65**, 471.
—— (1953b). *Science*, **118**, 332.
—— and West, G. B. (1953). *J. Physiol.*, **120**, 528.
Rocha e Silva, M., Bier, O., and Aronson, M. (1951). *Nature, Lond.*, **168**, 465.
Rothschild, A. M., and Rocha e Silva, M. (1954). *Brit. J. exp. Path.*, **35**, 507.
Wyman, L. C. (1929). *Amer. J. Physiol.*, **89**, 356.

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THE EFFECTS OF MECAMYLAMINE IN THE CAT AS MODIFIED BY THE ADMINISTRATION OF CARBON DIOXIDE

BY

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A series of cats, anaesthetized with chloralose, was given intravenously a sufficient quantity of mecamylamine to produce hypotension and a 50% neuromuscular block. Inhalation of carbon dioxide then produced a further depression of blood pressure and an increase in the neuromuscular blocking effect. It was shown that, following the administration of carbon dioxide, the plasma mecamylamine levels increased coincident with the increased pharmacological effects.

Since its introduction as a ganglion blocking agent, mecamylamine (3 methyl amino*iso*camphane hydrochloride) has been known to possess a weak blocking action at the neuromuscular junction (Stone, Torchiana, Navarro and Beyer, 1956). Relatively large doses of the drug are required before this action appears, and it is unlikely that, under normal circumstances, this property will present any clinical problems. Nevertheless, in the experimental animal the effects of mecamylamine at the motor end plate may help to throw light on its mode of action.

During the administration of mecamylamine there is evidence that considerable quantities are stored in the tissues whereas the plasma level remains low (Milne, Rowe, Somer, Meuhrcke, and Crawford, 1957). The present study indicates that when carbon dioxide is inhaled by a cat previously given mecamylamine, the plasma level of mecamylamine is raised, the neuromuscular block is potentiated and the hypotensive effect is intensified.

METHOD

Nine healthy but otherwise unselected cats were anaesthetized with chloralose (80 mg./kg.) after induction with ethyl chloride and ether. A tracheal cannula was inserted, and artificial respiration established by means of an "Ideal" respiration pump. This method was employed to avoid the risk of hypoxia during neuromuscular block and to facilitate the administration of carbon dioxide. Neuromuscular blocking activity was assessed using the nerve muscle preparation described by Brown (1938). In these experiments the tendon of the tibialis anterior muscle was isolated and connected to a flat spring myograph writing on a smoked drum. The lateral branch of the sciatic nerve was exposed in the thigh and shielded platinum electrodes applied to it. The stimulus was a rectangular pulse of 0.5 msec. duration applied at 10 sec. intervals and was maximal at a strength of 1 to 3 V. The blood pressure was measured from the common carotid artery with a mercury manometer. The intravenous injections were given into the right external jugular vein cannulated for the purpose.

The possibility of a direct depressant effect by mecamylamine on muscle was excluded by stimulating the tibialis anterior directly in one cat. When this occurred the muscle contracted normally even after mecamylamine had reduced the twitch by 50% on indirect stimulation.

Mecamylamine was given by a divided dose technique recommended by Paton (personal communication) so that the amount required to produce a standard degree of neuromuscular block could be more readily measured. An initial injection of 5 to 10 mg./kg. was given followed by equal increments until a 50% reduction in twitch size was obtained. This was taken as standard for these experiments and was attained with a total dose of 40 to 50 mg./kg. mecamylamine. When hypotensive and blocking activity had stabilized approximately 30 min. after the final mecamylamine injection, 10 or 20% (v/v), carbon dioxide was administered for periods of 10 to 30 min. Samples of blood and muscle and in some instances other tissues were taken immediately before and approximately 30 min. after mecamylamine administration, after the maximum effects of carbon dioxide and on recovery from these effects. The mecamylamine content of these samples was estimated by the method described by Baer, Paulson, Russo, and Beyer (1956) modified from Brodie, Udenfriend, Dill,

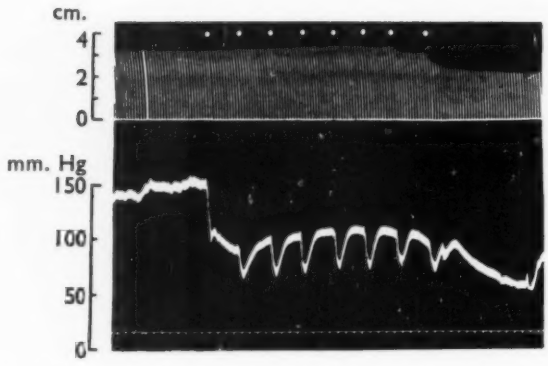

FIG. 1.—Record of the contractions of the tibialis anterior muscle and of the blood pressure before and after repeated intravenous injection of 5 mg./kg. of mecamylamine (at the white dots) into a cat anaesthetized with chloralose. Time, 30 sec.

and Downing (1947). In some animals the renal arteries were ligated to prevent loss of the drug in the urine during the experiment.

RESULTS

After the intravenous administration of 5 to 10 mg./kg. of mecamylamine there was an immediate and abrupt fall in blood pressure (Fig. 1) associated with bradycardia. This extreme hypotension was transient, and partial recovery, preceded by an increase in pulse rate, was observed within 1 min. A similar sequence of events succeeded each injection of mecamylamine. When the blood pressure was ultimately stabilized the degree of hypotension was invariably less marked than that initially observed.

Under normal circumstances there is an immediate rise in blood pressure when carbon dioxide is given to a cat, and this rise is sustained until the carbon dioxide is discontinued, when it falls sharply. In addition there is usually, but not invariably, a slight depression of the muscle twitch which is slower in onset than the hypertension and only gradually recovers when the inhalation of carbon dioxide is abandoned (Fig. 2).

After mecamylamine, instead of a rise the inhalation of carbon dioxide produced an acute fall in blood pressure (-34%, $P<0.001$); this fall was maintained until the administration of carbon dioxide was discontinued (Fig. 4), when the blood pressure rose and then gradually returned to the level at which it was stabilized after the injections of mecamylamine. Repeated administrations of carbon dioxide for short intervals became progressively less effective as the time interval lengthened after the injections of mecamylamine.

The preliminary intravenous injections of mecamylamine were followed almost at once by

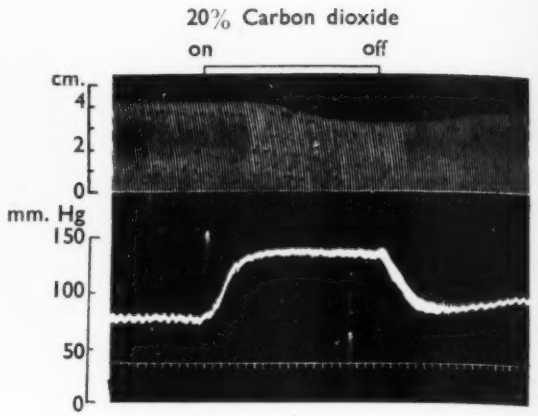

FIG. 2.—Record of the contractions of the tibialis anterior muscle and of the blood pressure before, during, and after the inhalation of 20% carbon dioxide by a cat anaesthetized with chloralose. Time, 30 sec.

a slight potentiation of the muscle twitch which was replaced by depression as the dosage was increased (Fig. 1). A depression to approximately 50% of normal was obtained with doses between 40 and 50 mg./kg. The inhalation of carbon dioxide increased the depression in all experiments (twitch decreased 48% $P<0.001$) as demonstrated in Fig. 3, and sometimes complete block was achieved. Since the effects of mecamylamine can be observed for some hours it was possible to demonstrate successive potentiation and recovery by intermittent administrations of carbon

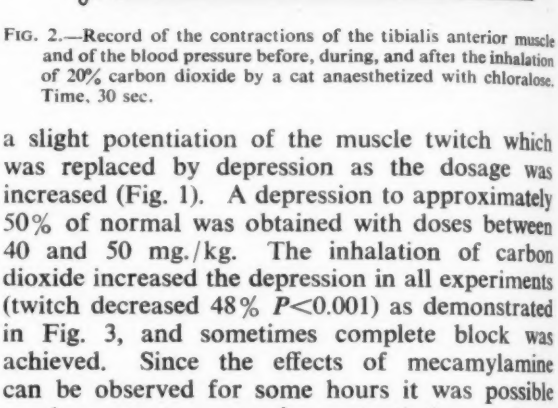

FIG. 3.—Record of the contractions of the tibialis anterior muscle and of the blood pressure in a cat anaesthetized with chloralose. *a*, Control records of the muscle twitch and blood pressure. *b*, 40 mg./kg. of mecamylamine was given intravenously 30 min. before the record was made. Note the effect of the inhalation of 20% carbon dioxide. Time, 30 sec.

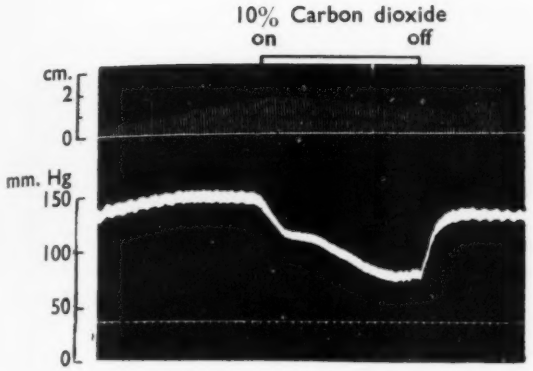

FIG. 4.—Record of the contractions of the tibialis anterior muscle and of the blood pressure in a cat anaesthetized with chloralose. 30 min. before this record was started, 40 mg./kg. of mecamylamine was given intravenously. Shortly afterwards the cat inhaled 10% carbon dioxide for a period and this record commences during the recovery from this procedure. A further inhalation of 10% CO_2, for a period shown above the tracing, again decreased the muscle twitch and lowered the blood pressure. Time, 30 sec.

dioxide for short intervals (Fig. 4). As with the effects on blood pressure, the potentiation of the neuromuscular block became less obvious as time passed after the administration of mecamylamine.

The mecamylamine content of liver, lung, spleen, kidney, brain, and muscle was estimated in several animals and the plasma and erythrocyte content in all. The lungs, liver, kidney, and spleen contained more mecamylamine than did blood, brain, and muscle; the amounts were roughly comparable to those found previously in the rat (Milne *et al.*, 1957).

Unfortunately, there are major technical difficulties in connexion with the extraction of mecamylamine from tissues, and since the reaction used is non-specific for secondary amines (Baer *et al.*, 1956) the tissue levels obtained varied considerably. In addition, the drug levels in the organs fell rapidly during the individual experiments, probably because of redistribution after the initial high concentration associated with intravenous injection (Milne *et al.*, 1957). The rate of fall in lung, liver, and kidney concentrations was so rapid that the influence of carbon dioxide on the mecamylamine content of these tissues could not be assessed. An attempt to minimize this complication was made by ligating the renal vessels; but even these experiments did not give clear-cut answers. A second difficulty was that repeated biopsies of the same organ were required; and it was hard to be sure that later samples were derived from organs with a satisfactory blood supply corresponding to the normal physiological state. The plasma and erythrocyte content alone yielded consistent and reproducible results, free from the foregoing criticisms.

In 17 separate estimations in nine cats given mecamylamine the average plasma mecamylamine content was 13.0 ± 5 μg./ml. After administration of carbon dioxide, the average rise in plasma mecamylamine was 5.0 μg./ml., with a standard deviation of ± 0.8 μg./ml. This increase was significant because it was consistent for each animal and the "t" test for paired values gave a P value <0.001. Since the erythrocyte content remained unchanged, the ratio of the content in erythrocytes to that in plasma fell from 1.12 to 0.83 ($P<0.001$).

DISCUSSION

It has been established that when cats previously given mecamylamine were ventilated with carbon dioxide the plasma level of the drug was raised substantially. Previous work (Milne *et al.*, 1957) has shown that the mecamylamine content of the liver, lung, kidney, and spleen becomes considerably higher than that of the blood, within minutes of its administration. Presumably the additional quantity demonstrated in the blood under the conditions of the experiments is mobilized from these organs by the action of carbon dioxide. This presumption is supported not only by the fact that the mecamylamine content of the organs fell during the experiments, but also by the established effects of *p*H on protein binding of drugs (Goldstein, 1949).

At the same time it was shown that the hypotension and neuromuscular block that follow the use of mecamylamine were potentiated during ventilation with carbon dioxide. The associated rise in plasma mecamylamine concentration may help to explain these effects. It is possible that mecamylamine is pharmacologically active only in the extracellular phase as has been postulated for hexamethonium and other ganglion blockers (Paton and Zaimis, 1952). If this is so, the high organ content of mecamylamine could serve as an inactive reservoir from which the drug becomes available in appropriate circumstances such as the presence of excessive carbon dioxide. This explanation would account for the smooth and sustained action curve of the drug as well as the potentiation of neuromuscular block and the extension of the hypotensive effect.

Although a rise in plasma mecamylamine levels has been demonstrated during carbon dioxide inhalation it does not follow that the increased hypotension produced is necessarily due to ganglionic blockade. If the autonomic ganglia are already paralysed completely, increased con-

centrations of mecamylamine will not produce any greater effect at these sites. An additional action must be postulated, and the transient bradycardia and hypotension that follow the intravenous injection of mecamylamine draw attention to one possibility—namely, a direct depressant action of the drug on the heart itself. This interpretation was suggested by the work of Bennett, Tyler, and Zaimis (1957), who have shown that in the isolated perfused heart the administration of mecamylamine can reduce the force of contraction and produce cardiac arrest.

It is, of course, conceivable that the additional hypotension was the result of a peripheral action of carbon dioxide on the vascular bed in the absence of a central effect of the gas, mecamylamine having inhibited the vasoconstrictor response by the paralysis of sympathetic ganglia. Support for this hypothesis is provided by the observation that potentiation of the hypotensive response could be obtained with carbon dioxide in cats during total spinal block (Payne, unpublished observation) and by Goodman and Gilman (1955), who claim that a similar depression occurs in man if carbon dioxide is administered during spinal anaesthesia or after extensive sympathectomy.

It was also observed that the moderate neuromuscular block obtained with mecamylamine was potentiated when the animal was exposed to carbon dioxide, and it is reasonable to conclude that this increase in block is due to the rise in the mecamylamine level of the plasma caused by the carbon dioxide. It could be argued, however, that the further depression of the muscle twitch is a direct effect of carbon dioxide and unrelated to the plasma level of mecamylamine. This is unlikely because, although carbon dioxide could depress the individual muscle twitch in the cat (Fig. 2), the effect was slight compared with that in the experiments described.

When cats previously given mecamylamine intravenously are ventilated with carbon dioxide there was a rise in the plasma concentration of mecamylamine and a decrease in the erythrocyte/plasma ratio.

Carbon dioxide inhalation under these conditions also potentiated the hypotensive and neuromuscular blocking properties of mecamylamine. These effects are probably directly related to the rise in plasma concentration.

The mecamylamine content of the lung, liver, spleen, and kidney was considerably higher than the plasma concentration. These organs presumably provide the source of the drug made available to the plasma by the action of carbon dioxide.

The authors are indebted to Professor W. D. M. Paton for facilities to carry out the experimental work and also for much valuable advice and criticism. Thanks are due to Dr. J. F. Merry, of Messrs. Merck, Sharpe and Dohme, for liberal supplies of mecamylamine, and to Miss Hedges, Mr. M. A. Crawford, and Mr. D. Green for their technical assistance. The financial cost of the work was met by a grant from the Dan Mason Research Foundation to one of us (J. P. P.).

References

Baer, J. E., Paulson, S. F., Russo, H. F., and Beyer, K. H. (1956). *Amer. J. Physiol.*, **186**, 180.

Bennett, G., Tyler, C., and Zaimis, E. (1957). *Lancet*, **2**, 218.

Brodie, B. B., Udenfriend, S., Dill, W., and Downing, G. (1947). *J. biol. Chem.*, **168**, 311.

Brown, G. L. (1938). *J. Physiol.*, **92**, 23P.

Goldstein, A. (1949). *Pharmacol. Rev.*, **1**, 102.

Goodman, L. S., and Gilman, A. (1955). *Pharmacological Basis of Therapeutics*, 2nd ed., p. 919. New York: Macmillan.

Milne, M. D., Rowe, G. G., Somer, K., Meuhrcke, R. C., and Crawford, M. A. (1957). *Clin. Sci.*, in the press.

Paton, W. D. M., and Zaimis, E. J. (1952). *Pharmacol. Rev.*, **4**, 219.

Stone, C. A., Torchiana, M. L., Navarro, A., and Beyer, K. H. (1956). *J. Pharmacol.*, **117**, 169.

Brit. J. Pharmacol. (1957), **12**, 461.

THE EFFECTS OF NICOTINE, HEXAMETHONIUM AND ETHANOL ON THE SECRETION OF THE ANTIDIURETIC AND OXYTOCIC HORMONES OF THE RAT

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The actions of nicotine, hexamethonium, and ethanol on the hypothalamo-hypophysial system have been investigated in the rat. The antidiuretic action of nicotine was not inhibited by ethanol, nor by doses of hexamethonium which were sufficient to block both its pressor and convulsant actions. Hexamethonium itself had an antidiuretic action the mechanism of which has been investigated. Nicotine caused a release of oxytocin into the blood which was not blocked by ethanol nor significantly reduced by hexamethonium. The results suggest that any synapse which exists at the supraoptic nuclei is dissimilar in its pharmacological properties to synapses at autonomic ganglia.

Antidiuretic hormone (ADH) and oxytocin are released in response to stimulation of the central end of the vagus and to stimulation of sensory nerve endings during coitus and suckling (see Harris, 1955). From the work of Pickford and her colleagues (Pickford, 1939, 1947) it appears likely that neurohormonal reflexes of this nature involve a synapse at the supraoptic nuclei at which acetylcholine acts as the chemotransmitter of afferent nerve impulses, the supraoptico-hypophysial tract constituting the efferent pathway for the release of hormones from the neurohypophysis. That the system is analogous to synapses at autonomic ganglia or the adrenal medulla is suggested by the finding that nicotine has an antidiuretic action which is abolished by hypophysectomy (Burn, Truelove and Burn, 1945). In this investigation an attempt has been made to block the antidiuretic and chloruretic actions of nicotine in the rat by means of the ganglion-blocking agent hexamethonium. A preliminary account of this work has been given (Bisset and Walker, 1953).

The action of ethanol as a blocking agent has also been investigated since it has been shown that, although it inhibits the release of ADH in response to intravenous injection of acetylcholine (van Dyke and Ames, 1951), emotional stress (Ames and van Dyke, 1952) and intracarotid infusion of hypertonic saline (Dicker, 1954), it does not block the antidiuretic action of nicotine in man (Eggleton, 1949).

A parallel investigation has been carried out to determine the concentration of oxytocin in jugular venous blood after the injection of hexamethonium and nicotine into rats under ethanol anaesthesia.

METHODS

Experiments on Water and Chloride Excretion

The antidiuretic and chloruretic activities of drugs were compared by using Burn's method of assay of pituitary (posterior lobe) extract (PPLE) (Burn, 1937). In two series of cross-over experiments, 16 rats were given 5 ml./100 g. of either warmed tap water or 10% ethanol (v/v) by stomach tube. The rats were divided into 4 groups of 4 and each group was placed in a separate metabolism cage. Excretion of urine was measured at intervals of 15 min. until three consecutive readings differed by less than 1.5 ml. (Taylor and Walker, 1951). The time to maximal rate of excretion, as defined by Burn (1937), was calculated for each group.

The following drugs were injected 10, 25, or 30 min. after the water load or ethanol: nicotine hydrogen tartrate 0.5 or 0.75 mg./100 g. subcutaneously; hexamethonium bromide 0.5 or 5.0 mg./100 g. intraperitoneally; PPLE (Pituitrin: Parke, Davis) 6.0 mU./100 g. subcutaneously or intraperitoneally.

Control groups received corresponding injections of saline.

Urinary chlorides were estimated by Volhard's method.

Assay of Oxytocin in Blood

Male rats, 200 g. to 300 g., were anaesthetized with 5 ml./100 g. of 15% to 20% (v/v) ethanol by

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stomach tube, supplemented, if necessary, with 1 to 4 ml. of 20% ethanol by intraperitoneal injection. As soon as surgical anaesthesia had been obtained, usually within 30 min., hexamethonium or nicotine was injected and, either 5 or 10 min. later, blood was collected from the cut external jugular veins. One minute before collection, heparin *B.P.*, 4.0 units/100 g., was injected into the saphenous vein.

As a result of further experiments on a non-specific factor occurring in blood extracts (see Results), the use of polythene syringes and beakers was adopted for the withdrawal and collection of blood.

Extraction and assay of oxytocin were carried out by the method of Bisset and Walker (1954). In every instance, except where the total activity was very small, extracts were treated with sodium thioglycollate (Van Dyke, Chow, Greep, and Rothen, 1942), and it was assumed that any activity persisting after this treatment was due not to oxytocin but to some non-specific factor. Non-specific activity was present principally in those experiments in which glassware was still in use to collect blood, and in these cases the concentration of oxytocin was estimated as the difference in activity before and after treatment with thioglycollate. The validity of this procedure was established by previous work (Bisset and Walker, 1954).

In the assays, Pituitrin (Parke, Davis) was used as the standard.

Results

Experiments on Water and Chloride Excretion

The Effect of Hexamethonium on the Antidiuretic and Chloruretic Actions of Nicotine.—Table I gives mean times to maximal rates of excretion. The time in control groups was 93 min. The effect of nicotine was to prolong the time to 151 min. There was no difference in effect between the two doses of 0.5 and 0.75 mg./100 g. In groups in which nicotine was preceded, at an interval of 20 min., by hexamethonium (0.5 mg./100 g.), the time was 142 min., which is not significantly less than in groups receiving nicotine alone. The conditions were similar to those in which hexamethonium abolishes the convulsant action of nicotine in the rat (Laurence and Stacey, 1952). Hexamethonium itself in a dose of 0.5 mg./100 g. had a small antidiuretic effect, prolonging the time to 109 min., which differs significantly from the control ($P = <0.001$). A tenfold increase in the dose of hexamethonium to 5.0 mg./100 g. prolonged the time to 167 min., which exceeds that observed after nicotine and is equivalent to the effect of 6.0 mU. PPLE/100 g. In groups in which the larger dose of hexamethonium was injected 5 min. before nicotine the time was increased to 204 min., which is significantly greater than after nicotine alone ($P = <0.001$) or hexamethonium alone ($P = <0.001$), suggesting not an antagonism but a summation of effects. The same dose of hexamethonium injected 5 min. before PPLE increased the time from 172 to 215 min. ($P = <0.01$ and >0.001), showing that hexa-

TABLE I

THE ANTIDIURETIC ACTION OF HEXAMETHONIUM, NICOTINE, AND PPLE IN THE RAT

Doses of drugs are expressed as quantities/100 g. of body weight. Hexamethonium (0.5 mg.) was given 10 min., hexamethonium (5.0 mg.) 25 min., and nicotine and PPLE 30 min., after the water load. PPLE = pituitary (posterior lobe) extract [Pituitrin].

Drug	Time to Maximal Rate of Excretion (min.)		
	Mean	S.E.	No. of Groups
Saline (control)	93	1·19	24
Nicotine (0·5 or 0·75 mg.)	151	2·47	20
Hexamethonium (0·5 mg.) + nicotine	142	4·35	4
Hexamethonium (0·5 mg.)	109	2·02	4
,, (5·0 ,,)	167	4·16	16
PPLE (6·0 mU.)	172	4·21	12
Hexamethonium (5·0 mg.) + nicotine	204	3·04	20
Hexamethonium (5·0 mg.) + PPLE	215	13·28	4

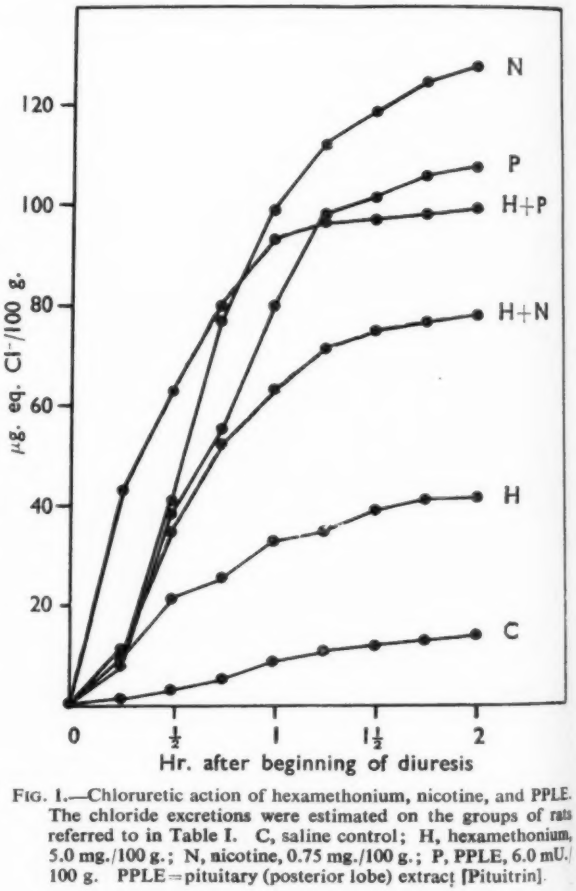

FIG. 1.—Chloruretic action of hexamethonium, nicotine, and PPLE. The chloride excretions were estimated on the groups of rats referred to in Table I. C, saline control; H, hexamethonium, 5.0 mg./100 g.; N, nicotine, 0.75 mg./100 g.; P, PPLE, 6.0 mU./100 g. PPLE = pituitary (posterior lobe) extract [Pituitrin].

methonium augments the antidiuretic action of nicotine and PPLE to a similar degree.

In the same experiments rates of urinary excretion of chloride were determined, with the results shown in Fig. 1. Both nicotine and PPLE produced a large increase in chloride excretion, but hexamethonium, although equipotent to both these drugs in its antidiuretic action, produced only a fraction of their chloruretic effect. In groups which received both hexamethonium and nicotine, the total chloruretic effect was intermediate between their effects when given separately. Hence hexamethonium increases the antidiuretic action of nicotine but reduces its chloruretic action. The fact that hexamethonium does not reduce the chloruretic action of PPLE suggests the possibility that its antagonism towards nicotine is due, not to a peripheral renal or vascular effect, but to a central inhibition of the release of a chloruretic factor or factors from the neurohypophysis.

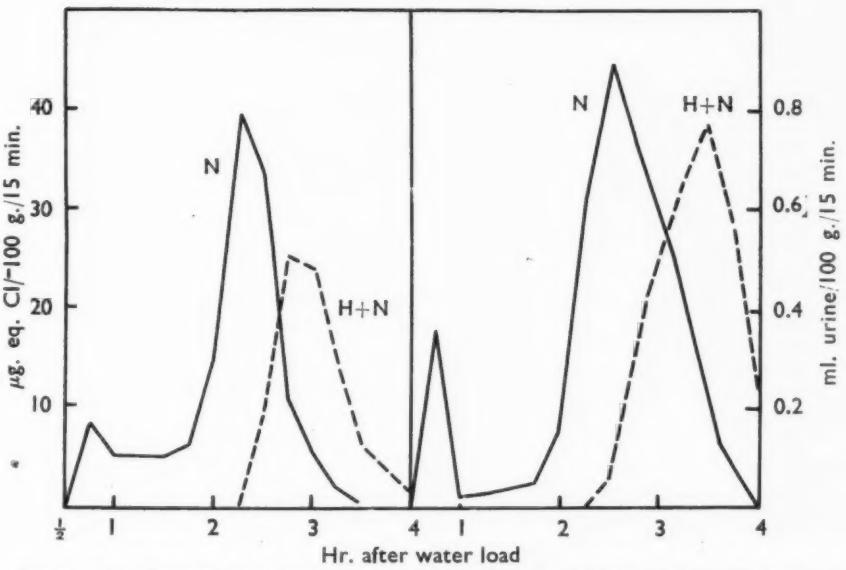

FIG. 2.—The effect of hexamethonium on the chloruretic and antidiuretic actions of nicotine. H, hexamethonium, 5.0 mg./100 g.; N, nicotine, 0.75 mg./100 g.

Fig. 2 compares the course of water and chloride excretion after nicotine alone (N) and nicotine and hexamethonium (H+N). Burn *et al.* (1945) observed that two peaks occur in a water diuresis curve after nicotine. Fig. 2 confirms this observation and shows also that two similar peaks occur in the curve of chloride excretion. The first peak, which in each case is reproducible by tyramine, and is attributed to the pressor action of nicotine, is abolished by hexamethonium; that this drug does in fact block the pressor action of nicotine has been confirmed by direct measurement of the blood pressure in a rat under similar experimental conditions. The second peak is shifted to the right, showing that the antidiuretic effect is increased. Fig. 2 also shows that the excretion of chloride is less after hexamethonium. The average total chloride excretion was 157 μg. eq. Cl^-/100 g. after nicotine alone and 86 μg. eq. Cl^-/100 g. after hexamethonium and nicotine. A correction for the amount of chloride excreted during the period of the initial pressor peak in the experiments with nicotine alone amounts to only 27 μg. eq. Cl^-/100 g., reducing the total to 130 μg. eq. Cl^-/100 g. Reduction of the chloruretic action of nicotine by hexamethonium cannot, therefore, be explained solely by abolition of a pressor effect.

The Antidiuretic Action of Hexamethonium.—Three possible mechanisms were investigated. (1) Delayed absorption of water from the intestine. (2) Effect on renal circulation due to a fall in systemic blood pressure. (3) Paralysis of the autonomic nerve supply to the bladder.

To test the first possibility, the following experiment was carried out. Sixteen rats, divided into two groups A and B of total weight 2,410 and 2,480 g. respectively, were given 5 ml./100 g. of warm tap water. Twenty-five minutes later each rat in group A was given a control injection of saline, and in group B hexamethonium 5.0 mg./100 g. intraperitoneally. Ninety-five minutes later, by which time the maximal rate of excretion and therefore of absorption of the water should have been attained, the rats were killed by decapitation and the urethras quickly ligated to prevent escape of urine. The abdomens were opened and, after ligation of the oesophagus, terminal ileum and neck of the bladder, the gastro-intestinal tract and the bladder were removed from each rat and weighed. The results are given in Table II. The mean weight of the gastro-intestinal tract in the control group A was 9.0 g. ± 0.64, compared with 10.1 g. ± 0.32 in the test group B: the difference is not significant. The total weight of the bladders in group A was 3.75 g. and in B 1.64 g. At the end of the experiment when the rats were killed.

TABLE II

THE EFFECT OF HEXAMETHONIUM ON ABSORPTION OF WATER FROM THE GUT

Group A, controls. Group B, hexamethonium 5·0 mg./100 g. 25 min. after water load.

Group	Wt. of Gastro-intestinal Tracts (g.)	m.	S.E.
A	8·7 7·7 7·7 8·9 7·5 7·9 11·4 12·2	9·0	0·64
B	9·5 9·9 10·8 10·2 9·2 9·2 10·5 11·8	10·1	0·32

group A had passed a total of 54.2 ml. urine and group B 2.5 ml. It is clear that the antidiuretic effect of hexamethoniuum in group B was not due to delay in the absorption of the water, nor was it associated with passive distension of the bladder.

To test the second and third possibilities, blood pressure was recorded simultaneously with urine flow in a rat under ethanol anaesthesia. As the bladder was cannulated in this preparation, any antidiuretic effect observed could not have been due to paralysis of its autonomic nerve supply. It was found that each intravenous injection of hexamethonium caused an inhibition of diuresis with a concomitant fall of blood pressure. If the fall of systemic blood pressure after hexamethonium is causally related to the antidiuretic effect, it might act either by a peripheral action on glomerular filtration in the kidney or indirectly by release of ADH from the neurohypophysis.

TABLE III

THE ANTIDIURETIC AND CHLORURETIC ACTIONS OF ETHANOL, NICOTINE, AND PPLE

Doses of drugs are expressed as quantities/100 g. of body weight. Nicotine and PPLE were given 30 min. after water or ethanol.

Drug	Time to Maximal Rate of Excretion (min.)			Total Chloride Excretion (μg. eq. Cl^-/100 g.)		
	Mean	S.E.	No. of Groups	Mean	S.E.	No. of Groups
Ethanol (10% v/v; 5·0 ml.)	95	7·0	4	49	9·6	4
Water (5·0 ml.) + nicotine (0·75 mg.) ..	146	2·9	4	200	12·6	4
Ethanol + nicotine ..	187	5·7	8	149	13·4	8
Water + PPLE (6·0 mU.)	144	2·7	6	192	14·9	6
Ethanol + PPLE ..	161	6·2	6	198	13·6	6

The Effect of Ethanol on the Antidiuretic and Chloruretic Actions of Nicotine.—A series of experiments was carried out in which the water load was replaced by 10% v/v ethanol. The doses of nicotine and PPLE were the same as in the experiments with hexamethonium.

The results are given in Table III. The mean time to maximal rate of excretion in the control groups receiving ethanol alone was 95 min. The effect of replacing the water load with ethanol was to increase the time after nicotine from 146 to 187 min. ($P = <0.001$) and to reduce the total chloride excretion from 200 to 149 μg. eq. Cl^-/100 g. ($P = <0.02$): after PPLE the time was increased from 144 to 161 min. ($P = <0.05 > 0.02$), but there was no significant change in total chloride excretion.

It is possible, therefore, that ethanol, like hexamethonium, reduces the chloruretic action of nicotine by a central inhibitory effect. It increases the antidiuretic action of nicotine, although it is not itself antidiuretic, but as it similarly enhances the antidiuretic action of PPLE the effect is, in this case, probably a peripheral one.

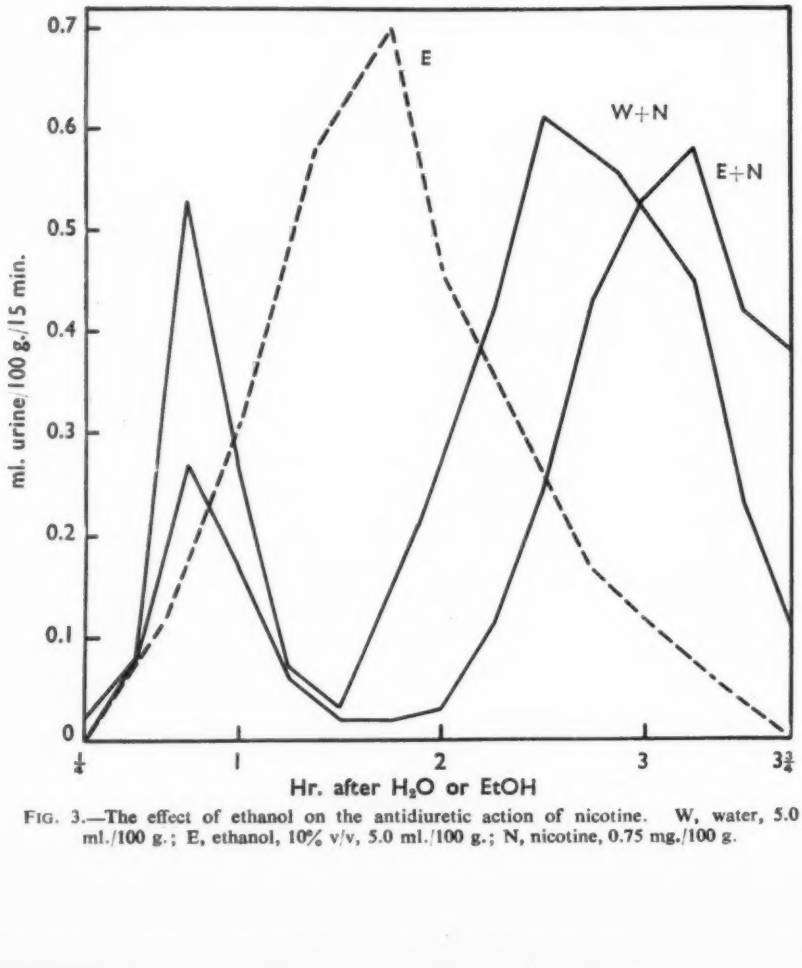

FIG. 3.—The effect of ethanol on the antidiuretic action of nicotine. W, water, 5.0 ml./100 g.; E, ethanol, 10% v/v, 5.0 ml./100 g.; N, nicotine, 0.75 mg./100 g.

Fig. 3 is included for comparison with Fig. 2. In the curves for both water and nicotine (W + N) and ethanol and nicotine (E + N) two peaks occur: the first peaks are coincident; in the ethanol and nicotine curve, however, the second peak is shifted to the right, showing that the antidiuretic action of nicotine has been potentiated by ethanol.

Assay of Oxytocin in Blood

Preliminary Experiments on a Non-specific Factor in Blood Extracts.—In a previous paper (Bisset and Walker, 1954) it was stated that in a number of experiments an oxytocic factor occurred in blood extracts which could not be identified with oxytocin, potassium or 5-hydroxytryptamine. Armstrong, Jepson, Keele and Stewart (1955) have since detected in human plasma a pain-producing substance (PPS) with oxytocic activity, which may be identical with bradykinin, and is formed when blood comes into contact with a water-wettable surface. The following experiment suggests that the non-specific factor encountered in our earlier experiments is a similar substance.

18.0 ml. blood was collected in a polythene syringe from the cut external jugular veins of four rats under ethanol anaesthesia. 9.0 ml. was introduced into a polythene beaker and 9.0 ml. into a glass beaker. The blood was allowed to stand for 10 min. The two samples were extracted by the usual method and assayed for oxytocic activity against PPLE. The activity of the first extract, prepared from the blood in the polythene beaker, and equivalent to 3.5 mU./ml., was abolished by sodium thioglycollate. The activity of the second extract amounted to 8.9 mU./ml., and this was reduced by treatment with thioglycollate to 5.1 mU./ml., the difference of 3.8 mU./ml. representing the content of oxytocin.

As a result of this experiment the use of polythene was adopted for the collection of blood samples. In none of 13 consecutive experiments following its use was non-specific activity encountered, although it has since recurred in occasional experiments.

Concentrations of Oxytocin in Jugular Venous Blood under Ethanol Anaesthesia.—The results obtained are set out in Table IV.

In control experiments under ethanol anaesthesia the average concentration of oxytocin in blood from the external jugular veins was 1.1 mU./ml. Non-specific oxytocic activity occurred in five of the six experiments in which glass was used to collect blood (Table IV, expts. 1 to 6) but was absent from the remaining five experiments in which glass was replaced by polythene.

TABLE IV

CONCENTRATIONS OF OXYTOCIN IN THE EXTERNAL JUGULAR VENOUS BLOOD OF RATS UNDER ETHANOL

The figures in parentheses give the volume of blood in ml./100 g. withdrawn in each experiment. The estimates for oxytocin are given in mU./ml. of jugular venous blood.

Expt. No.	Control	Nicotine	Hexamethonium + Nicotine	Hexamethonium
1	0·0	17·3 (3·1)	0·0 (1·3)	2·7 (1·3)
2	0·0	5·2 (1·6)	7·0	2·8 (1·6)
3	0·7	8·3 (2·7)	3·8	0·6 (0·9)
4	1·4	8·2 (2·0)	5·0 (1·6)	≯0·5 (1·8)
5	2·5 (2·1)	9·5 (1·7)	4·5 (1·4)	0·4 (1·5)
6	1·9 (2·6)	8·6 (2·2)	≯1·3 (0·9)	1·2 (1·7)
7	1·0 (1·7)	5·1 (2·0)	0·0 (1·1)	3·3 (1·2)
8	0·6 (1·9)	0·0 (1·6)	1·3 (1·0)	≯0·6 (1·2)
9	1·6 (1·4)	5·4 (1·6)	6·1 (1·2)	
10	1·4 (1·3)	8·2 (1·9)	3·3 (2·3)	
11	1·5 (1·8)	3·1 (2·9)	4·5	
12		2·1 (1·8)	8·5	
13		0·0 (2·2)		
14		3·4 (2·4)		
15		11·8 (2·2)		
16		1·3 (1·7)		
17		4·1 (1·7)		
18		7·2 (1·8)		
19		3·3 (1·3)		
20		8·6 (2·0)		
21		0·0 (2·0)		
22		4·7 (1·1)		
23		6·2 (2·2)		
24		5·3		
m	1·1	5·7	3·8	1·5
S.E.	0·23	0·82	0·79	0·43

The mean concentration after nicotine was 5.7 mU./ml. oxytocin. This value is significantly higher than the mean in the ethanol controls ($P = <0.001$). In three experiments (Table IV, expts. 8, 13, and 21) oxytocin was not detected. In experiment 16 two assays of oxytocic activity were carried out. In the first, the total activity was 9.3 mU./ml., but this was not demonstrably reduced by treatment with thioglycollate. The second assay was carried out three days later: the activity was 1.3 mU./ml., and this was abolished by thioglycollate. It is possible that the preparation used for the second assay was relatively insensitive to the non-specific factor. The experiment emphasizes the importance of submitting all active extracts to the thioglycollate test. In experiments 18 and 19 the extracts, when tested on the rat anaesthetized with ethanol, caused an increase in total chloride excretion.

After hexamethonium alone the mean concentration of oxytocin was 1.5 mU./ml., which was not significantly different from the control series. In the experiments in which nicotine was given 5 min. after hexamethonium the mean concentration of oxytocin in the blood was 3.8 mU./ml. This value does not differ significantly from that obtained after nicotine alone. The results show that under these conditions hexamethonium does

not liberate oxytocin, nor does it inhibit the release of oxytocin by nicotine.

ADH is known to be released by haemorrhage (Ginsburg and Brown, 1956) and this might apply also to oxytocin. In Table IV the volume of blood withdrawn in each experiment is shown. In the whole series, comprising both control and test groups, there is some degree of correlation between this volume and the concentration of oxytocin in the blood ($r = +0.46$). However, only in the case of the nicotine group is the mean volume of blood withdrawn greater than in the control group and the difference is not significant ($P = >0.05$). The differences in the mean concentrations shown for the various drugs are therefore likely to be due not to an accidental effect of haemorrhage but to the actions of those drugs on the neurohypophysis.

Discussion

The experiments reported in the first part of this paper showed that nicotine caused a profound antidiuresis and chloruresis in the rat. The antidiuresis was presumably due to release of ADH (Burn *et al.*, 1945), and the chloruresis might have been due to the release of oxytocin, since several workers have attributed the chloruretic effect of PPLE to its oxytocic fraction. The previous injection of hexamethonium or administration of ethanol did not inhibit the antidiuresis caused by nicotine, but did inhibit the chloruresis. Since neither of these drugs affected the chloruretic action of injected PPLE, the results suggested the possibility that they inhibited centrally the release by nicotine of oxytocin but not of ADH. This would mean in fact that under certain conditions the two hormones could be secreted independently by the neurohypophysis.

In order to approach the problem more directly, rats anaesthetized with ethanol were given the same injections of nicotine and hexamethonium as in the experiments on water diuresis, and samples of blood from the external jugular veins were assayed for oxytocic activity. It was found that the activity of the blood of animals which had received nicotine was significantly higher than that of the controls. Thus nicotine releases oxytocin from the neurohypophysis, and this release is not blocked by ethanol. However, the mean levels in the blood after nicotine alone were not significantly different from those after hexamethonium and nicotine, so that the hypothesis that hexamethonium inhibits the release of oxytocin by nicotine is not confirmed. The results suggest that the chloruretic action of nicotine is due not only to release of oxytocin but also to some other effect on renal function which is inhibited by hexamethonium.

In some experiments, assays of both hormones in the blood were carried out in an attempt to see if the ratio in which they were released was constant or variable. It appeared at first that the ratio of oxytocin to ADH was significantly higher after nicotine than in the controls, and these results have already been quoted by one of us (Walker, 1957). However, while the present paper was still in preparation, Ginsburg (1956) reported that Pituitrin, which was used in our experiments as the standard, had less antidiuretic activity in relation to its pressor and oxytocic potencies than did the international standard of PPLE, and that the activity varied greatly according to the particular rat used for the assay. The results of assays of ADH are therefore of doubtful value and they have been omitted from the tables. It is intended to repeat this part of the work using a more satisfactory standard.

It has been shown that hexamethonium does not block the release of oxytocin nor inhibit the antidiuretic action of nicotine in doses which are sufficient to block both the pressor and convulsant actions of this drug. However, the anticonvulsant action of hexamethonium is not to be taken as evidence of a central inhibitory effect, for Laurence and Stacey (1953) showed that it depends on blockage of the release of adrenaline. It is indeed possible that the drug fails to penetrate the blood-brain barrier. If, however, it does reach the anterior hypothalamus it must be concluded that any synapse which exists at the supraoptic nucleus is dissimilar in its pharmacological properties to synapses at autonomic ganglia. This is in agreement with the observations of Supek and Eisen (1953), who similarly failed to block the antidiuretic and chloruretic actions of nicotine in rats with a number of ganglion-blocking agents.

The ability of ethanol to produce a state of "functional neurohypophysectomy" (Dicker, 1954) evidently depends on the nature of the stimulus to the neurohypophysis. In this investigation, ethanol did not inhibit the antidiuretic action of nicotine, nor block the release of oxytocin. This is consistent with the finding of Ginsburg and Brown (1956) that the antidiuretic activity of blood during haemorrhage in rats under ethanol anaesthesia is only slightly lower than that under ether, pentobarbitone, or urethane.

This work was carried out during the tenure by one of us (G. W. B.) of a Scholarship for Training in Research Methods, for which he wishes to express his gratitude to the Medical Research Council.

References

Ames, R. G., and van Dyke H. B. (1952). *Endocrinology*, **50**, 351.

Armstrong D., Jepson J. B., Keele, C. A., and Stewart, J. M. (1955). *Nature, Lond.*, **174**, 791.

Bisset, G. W., and Walker. J. M. (1953). *Abstr. XIX int. physiol. Congr., Montreal*, 1953, p. 251.

—— —— (1954). *J. Physiol.*, **128**, 588.

Burn, J. H. (1937). *Biologica' Standardization*, 1st ed., p. 69. London: Oxford University Press.

—— Truelove, L. H., and Burn, I. (1945). *Brit. med. J.*, 1, 403.

Dicker, S. E. (1954). *J. Physiol.*, **124**, 464.

Eggleton, M. G. (1949). Ibid., **108**, 482.

Ginsburg, M. (1956). *Brit. J. Pharmacol.*, **11**, 245.

—— and Brown, L. M. (1956). Ibid., **11**, 236.

Harris, G. W. (1955). *Neural Control of the Pituitary Gland.* London: Edward Arnold.

Laurence, D. R., and Stacey, R. S. (1952). *Brit. J. Pharmacol.*, **7**, 80.

—— —— (1953). Ibid., **8**, 62.

Pickford, M. (1939). *J. Physiol.*, **95**, 226.

—— (1947). Ibid., **106**, 264.

Supek, Z., and Eisen, V. (1953). *Arch. int. Pharmacodyn.*, **93**, 75.

Taylor, N. B. G., and Walker, J. M. (1951). *J. Physiol.*, **113**, 412.

van Dyke, H. B., and Ames, R. G. (1951). *Acta endocrinol.*, **7**, 110.

—— Chow, B. F., Greep, R. O., and Rothen, A. (1942). *J. Pharmacol.*, **74**, 190.

Walker, J. M. (1957). *The Neurohypophysis*, p. 221. London: Butterworths.

Brit. J. Pharmacol. (1957), **12**, 468.

THE TOXICITY OF DIAMINODIPHENOXYALKANES

BY

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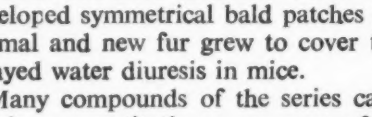
Representative members of a series of schistosomicidal diaminodiphenoxyalkanes were examined for their toxic effects in laboratory animals. Primary amino-derivatives were, in general, more toxic than secondary methylamino-compounds; tertiary compounds were less toxic than either. Large doses given by mouth or by injection to mice or rabbits produced intravascular haemolysis; the haemoglobin and erythrocyte counts began to increase again about a week after the dose. Mice which had been given large doses of diaminodiphenoxyalkanes developed symmetrical bald patches 2 to 3 weeks after treatment. The exposed skin appeared normal and new fur grew to cover the hairless areas in about 6 weeks. Large doses of drug delayed water diuresis in mice.

Many compounds of the series caused visual impairment when given to cats. In order to obtain a quantitative assessment of retinotoxic potency, a method was devised in which the ability of the retina of the frog to resynthesize rhodopsin was measured in treated and control animals. Compounds that produced blindness in cats also inhibited rhodopsin synthesis in frogs; the most toxic compounds were primary amines. Treatment with some tertiary amines caused retinal damage if the drugs were given for long periods. Diaminodiphenoxyalkanes may perhaps interfere in some way with the biological activity of vitamin A. However, toxic effects on the hair and retina were not prevented by supplements of synthetic or natural vitamin A.

The activity of a series of diphenoxyalkanes (I) against experimental schistosomiasis in mice has been reported by Standen and his colleagues (Raison and Standen, 1954, 1955; Caldwell and Standen, 1956; Standen and Walls, 1956).



Compounds of the same general type have been examined independently by Collins, Davis, and Hill (1954), and Edge, Mason, Wien, and Ashton (1956) have described the toxic effects of some members of the series upon the eyes of laboratory animals. The present paper deals with the toxic actions of representative members of the series tested by Standen.

A large number of compounds was studied, but for simplicity this account is concerned mainly with primary, secondary, and tertiary derivatives of diaminodiphenoxyheptane. The toxic actions of these compounds are typical of the series. The serial numbers and chemical structures of these compounds are: 153C51—R = NH_2; 413C52—R = NHMe; 27C53—R = NMe_2; 296C53—R = NEt_2; 252C53—R = $N(CH_2.CH_2OH)_2$.

METHODS

Diphenoxyalkanes.—Compounds were prepared and supplied as hydrochlorides by Drs. Caldwell, Raison, and Walls. Aqueous solutions became cloudy on dilution because of hydrolysis; solutions prepared for injection were freed from turbidity by adding a small amount of dilute HCl. Warming was necessary to dissolve some of the compounds with long chains. When large doses of drugs were given by mouth, suspensions were prepared with compound powder of tragacanth.

Toxicity to Mice.—Groups of 10 mice, weighing 20 to 25 g., were injected intravenously with 1% aqueous solutions of the drugs. Injections were given in 6 to 8 sec. Mortality was observed for three days; the LD50 of each compound was estimated graphically. Further experiments were made on groups of 10 or 20 mice which were given large doses (500 to 1,000 mg./kg.) of selected compounds by mouth on two or three successive days.

Effects on the Blood.—Haemoglobin determinations and red and white cell counts were made at intervals

during the treatment of mice, rabbits, and monkeys with compounds given by mouth, or by intravenous or subcutaneous injection. The method of Evelyn and Malloy (1938) was used to estimate methaemoglobin and sulphaemoglobin in whole blood. The serum was separated and examined spectroscopically.

Effect upon the Hair.—Loss of hair in mice caused by compounds of the series was assessed three weeks after treatment by awarding "scores" to individual mice and averaging the scores for groups of 10 or 20 animals. A score of 1 was awarded for partial loss, and 2 for complete loss of fur on head, back, or belly; a completely bald mouse would therefore score 6.

A number of supplements to the diet was given in attempts to antagonize the action upon the fur. Substances were incorporated in the diet, or were given by stomach tube or by subcutaneous injection, beginning before treatment with the diphenoxyalkane, and continuing throughout the experiment. Inositol, sodium *p*-aminobenzoate, calcium pantothenate, vitamin A (synthetic), fish liver oil concentrate, an alcoholic extract of liver (Armour's fraction 2), and various oils and fats were tested.

Effect upon the Excretion of Urine.—Groups of 10 mice were given doses of drugs by mouth; each animal received 0.5 ml. of solution. Urine was collected at intervals in a metabolism cage and the volumes excreted compared with those from mice given 0.5 ml. of water instead of drug.

Effect upon the Eyes of Cats.—Doses of selected compounds were given to cats by mouth or by subcutaneous injection. Acuity of vision was tested by the response of the cat to visual stimuli and by its behaviour when placed on a table or on the floor of a room. The fundi were examined with an ophthalmoscope, and histological preparations of some of the retinas were made.

Effect upon Rhodopsin Synthesis in Frogs.—The method used is related to one described by Zewi (1941) in a study of the actions of atropine and pilocarpine. Groups of 3 to 5 frogs (*Rana temporaria*) were given doses of selected compounds at 9 a.m., 5 p.m., and at 9 a.m. the following morning. The drugs were injected into the ventral lymph sac, or were given by stomach tube. The frogs were kept in about 1 in. depth of water in glass jars with perforated perspex lids. Immediately after the second dose the jars were placed in a white box so that the frogs were 20 in. below the central parts of two "warm white" fluorescent tubes (80 w.). The illumination at the bottom of each jar, measured with a light-meter, was 250 to 300 f.c. After being left for 17 hr. under the light, most of the retinal rhodopsin had been bleached. The jars were placed in complete darkness in a black box for 2 hr. so that resynthesis of rhodopsin could occur. The frogs were decapitated, the heads washed free from blood and the retinas removed from the eyes by the light of a dark-room lamp (Wratten, series 2). The retinas from each group of three frogs were added to 2.5 ml. of McIlvaine's phosphate buffer (*p*H 4.6) in a centrifuge tube and stirred to free the rods by inserting a stainless steel ball and moving the tube between the poles of a strong magnet. The tube was centrifuged for 20 min. in an M.S.E. angle centrifuge at 4,000 r.p.m., and the buffer discarded. The visual pigment was extracted from the rods with two portions of 1.5 ml. of 2% digitonin solution, the tubes being well stirred with the ball and centrifuged for 20 min. for each extraction. All these operations were performed in the dark. The combined extracts were made slightly alkaline by the addition of 0.2 ml. of saturated aqueous borax solution and transferred in the dark to the cell of a spectrophotometer for the measurement of absorption at 502 mμ. The extract was then bleached by exposure for 5 min. to the light of a 150 w. bulb at a distance of 15 cm., and the absorption measured again. The difference between the readings was proportional to the amount of light-sensitive pigment in the retinal extract. Control frogs which had been injected with 0.6% saline instead of drugs were used in each experiment, and the amount of rhodopsin resynthesized by treated frogs was expressed as a percentage of the control value. In a comparison of this kind it was considered unnecessary to go to further lengths to obtain the visual pigment pure.

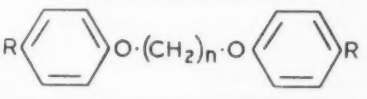
In some experiments, larger batches of 50 to 100 frogs were treated for longer periods and samples of three or five were taken from time to time to assess the effect of continued treatment and the rate of recovery when treatment ceased. Each group was exposed to standard conditions of light followed by darkness, alongside a group of normal controls treated with water or saline. A few experiments were made in which frogs were given large oral doses of synthetic or natural vitamin A (100,000 i.u./20 g. frog) before and during treatment with diphenoxyalkanes.

RESULTS

The acute toxicities to mice of a series of primary, secondary, and tertiary amino-derivatives are shown in Table I. The dose-response curves were almost parallel, and the figures in Table I

TABLE I

THE ACUTE INTRAVENOUS TOXICITY OF SOME DIPHENOXYALKANES TO MICE



Chain Length n	LD50 (mg./kg.)		
	R=NH$_2$	R=NHMe	R=N(Me)$_2$
5	140	180	120
6	100	71	46
7	71	220	250
8	110	56	72
9	110	240	84

have limits of error ($P=0.05$) of the order of 80 to 130%. Intravenous injection of large doses of primary amines caused death after a few minutes of convulsive movements. With the secondary and tertiary compounds convulsions were less marked, the main sign being flaccid paralysis of the hind limbs; respiration was not greatly affected in mice that did not die. The values of the LD50 of the primary amines tested were all of the same order. Secondary and tertiary amines with odd- and even-numbered carbon chains alternated in toxicity, those with 6 and 8 carbon atoms being more toxic than those with 5, 7, or 9. It is of interest that the schistosomicidal activity of tertiary methylamines also alternates, but in this instance compounds with even numbers of carbon atoms are a little less active than those with odd numbers (Raison and Standen, 1955).

When given by mouth, the drugs were tolerated by mice in single doses of 0.5 to 2 g./kg. When doses of 500 mg./kg. or more were given orally on 2 or 3 successive days, mice lost weight, the nose, paws, and tail became bluish in tint, and the fur was sometimes shed in symmetrical patches. Weight began to increase again about a week after treatment. Secondary amines were more prolonged in their action than the corresponding primary amines; the tertiary amines were less toxic than either when given by mouth.

Effect on the Blood.—The blue tint of the nose and paws of a mouse treated with large doses of diphenoxyalkanes was caused mainly by the presence of brownish-grey metabolites of the drugs in the blood and tissues. However, there was also a marked effect on the number of red cells, which decreased to about one-half the normal value after three oral doses of 500 mg./kg. The haemoglobin followed the erythrocyte count; both began to increase again at the end of a week. Fig. 1 shows the effect of heptane derivatives in the mouse and rabbit. Intravenous injection caused a very rapid decrease in red cells, and it was clear that the drugs, or their breakdown products, caused intravascular haemolysis. The fragility of the rabbit's erythrocytes was increased; the serum was stained reddish-brown and showed the absorption spectrum of haemoglobin. Small amounts of met- and sulphaemoglobin were present in the serum of treated mice and rabbits, but there was no significant change in serum bilirubin.

A short-lived polymorphonuclear leucocytosis followed the destruction of erythrocytes (Fig. 1). Blood films showed the presence of many immature red cells, and histological preparations made by Dr. D. Trevan from young rats which had

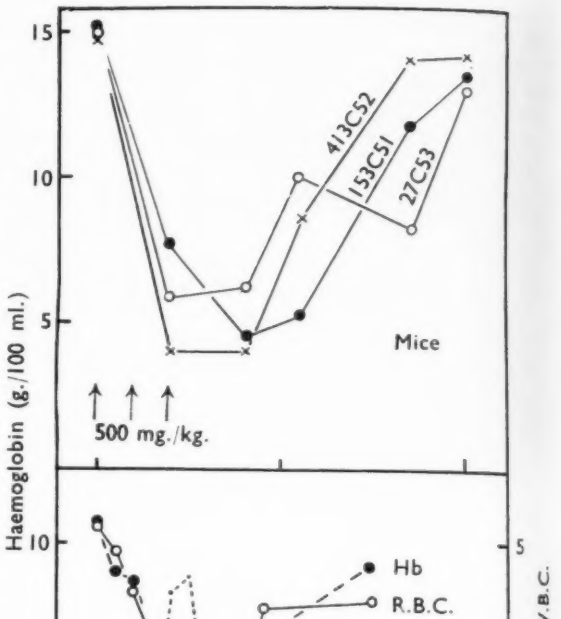


FIG. 1.—The effect of oral doses of diphenoxyalkanes on the blood of the mouse and rabbit. Ordinates for R.B.C. are in units of 10^6, and for W.B.C. in units of 10^4. Each point in the upper graph is the mean of two mice. The lower graph is from a rabbit weighing 4.3 kg.

been given oral doses of diphenoxyalkanes for 30 to 60 days showed that some of these new erythrocytes were derived from islands of erythropoietic tissue in the red pulp of the spleen. The bone marrow of most of the rats was normal.

Loss of Hair.—About 2 weeks after the administration of 2 or 3 large doses mice began to lose their hair. The remaining fur became silky and tousled; this change was also present in mice which were not sufficiently affected to show bald patches. A change of texture of coat occurred in rats, but no actual loss of hair was observed in rats, rabbits, cats, or monkeys. In mice, bare patches usually appeared on the head, back, and flanks, and the fur on the abdomen became sparse and fluffy (Fig. 2). Loss of hair was invariably symmetrical. The underlying skin was normal in appearance, and the claws and vibrissae were not noticeably affected. When "scores" were

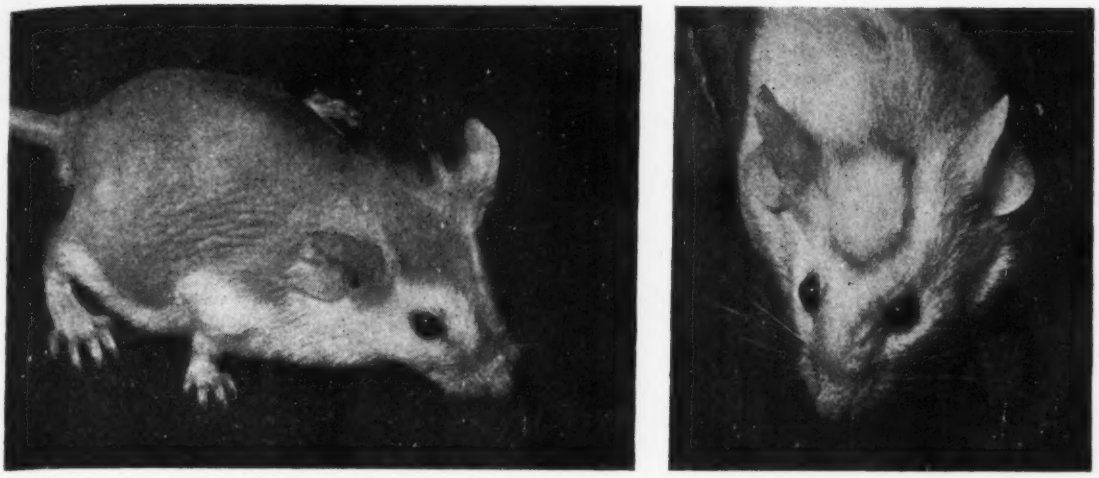


FIG. 2.—Examples of loss of hair in mice given 3 oral doses of 500 mg./kg. of a diphenoxyalkane 3 weeks previously. One mouse shows extensive loss of fur over the back and head; the other has a symmetrical pattern on the head only.

awarded to mice at the time of maximal effect (3 weeks after treatment), it was seen that the tertiary amines were the least, and the primary amines the most, toxic. In a typical experiment with the heptane derivatives the mean scores for groups of 10 mice were: 153C51—2.5; 413C52—1.7; 27C53—0.7. The ethyl compounds 296C53 and 252C53 were more toxic than the methyl compound 27C53. It is probable that the drug affected follicles which would have formed the coat in 3 weeks' time; these failed to develop, leaving bald patches when the older hairs had been shed. Subsequent crops of follicles were not affected, and 6 weeks after treatment the hair had grown again.

Phenolic compounds such as *p*-aminophenol and metol, which are possible metabolites of the diphenoxyalkanes, had no action of this kind on the fur of mice. Dietary deficiencies are a known cause of alopecia and therefore experiments were made in which supplements were given as possible antagonists to the action of 413C52. Inositol (500 mg./kg. daily) given by mouth or by subcutaneous injection, calcium pantothenate (500 mg./kg. daily), synthetic vitamin A acetate (1,000 i.u./mouse daily), or fish liver oil concentrate containing natural vitamin A (70,000 i.u./mouse daily) did not prevent loss of hair. Liver extract in enormous doses (20 to 200 mg./mouse daily) partially prevented loss of weight, but did not prevent loss of hair; sodium *p*-aminobenzoate (25 mg./kg. daily) increased the general toxicity of the drug and had no action on the loss of hair. Lard given at 10% in the diet prevented hair-loss in one experiment, but we were unable to repeat this in subsequent tests. No antagonistic action was shown by fats selected for their saturated or unsaturated fatty acid content (iodine values 8.8 to 86.1).

Inhibition of Diuresis.—Mice given large doses of primary, secondary, or tertiary amines by mouth excreted less urine than normal controls. Primary amines were the most, and tertiary amines the least, toxic in this respect (Fig. 3). Small doses of the primary amine 153C51 caused slight stimulation of the urine flow; doses of 75 mg./kg. or over caused inhibition. An effect on the urine flow was also observed when the dose was given by subcutaneous or intravenous injec-

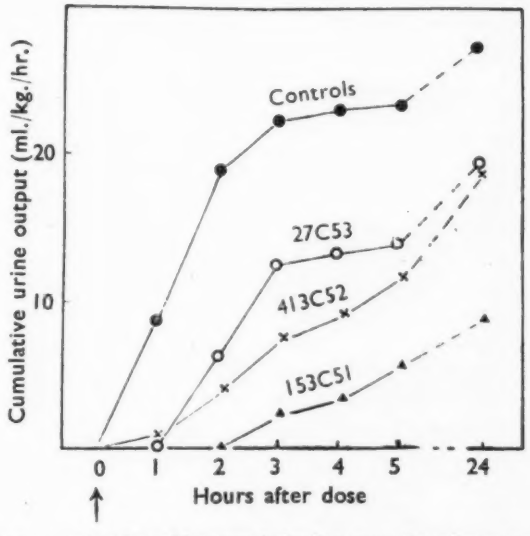


FIG. 3.—The effect of heptane derivatives upon the urine output of mice. Groups of 10 animals were given doses of 1 g./kg. orally. The curves show the means of two experiments.

tion. No significant histological changes were seen in the kidneys of animals which died after treatment with this drug.

Effects on the Eye.—Large and repeated doses of diphenoxyalkanes given by mouth had no apparent action upon the eyes of mice, rats, or guinea-pigs. On the other hand, many compounds when given orally had an effect on the eyes of cats. Within a few days the cat failed to respond to visual stimuli, refused to jump to the floor from a table, and explored the floor of the room with great care with its whiskers. The pupils were fully dilated, and failed to react to light. Examination of the fundus showed narrowing of the blood vessels and sometimes pallor of the optic disc. Pigmentation of the kind described by Edge *et al.* (1956) was not observed.

Sections of retinas showed areas of damaged pigment epithelium and affected rods. Many of the cells of the pigment epithelium were swollen and the pigment granules were distributed throughout the cytoplasm instead of being arranged in an orderly manner at the inner borders of the cells. In some areas the pigment in the epithelium was absent. Other layers of the retina were normal in appearance. The optic nerves were also examined and no abnormality was found.

Toxic effects on the retina were easy to obtain with primary and secondary amines; more prolonged treatment with large doses was necessary to get effects from tertiary methylamines such as 27C53. No blindness was produced by prolonged treatment with 296C53 or 252C53 by mouth.

The extent of blindness in cats is not easy to evaluate, and the experiments on frogs were designed to give a more objective measurement of the degree of toxicity of a drug to the retina. The results agreed very well with those obtained in cats.

TABLE II

RHODOPSIN IN THE RETINAS OF FROGS INJECTED WITH 153C51 AND EXPOSED TO PERIODS OF LIGHT AND DARKNESS

Groups of 3 frogs were given doses of 100 mg./kg. of 153C51 into the ventral lymph sacs at the beginning of the 24 hr. in the dark. The dose was repeated after 8 hr., and groups C and D were given a third dose after 24 hr. in the dark. Control groups were injected with 0.6% saline. Each rhodopsin figure was obtained by measuring the absorption of the retinal extract at 502 mμ before and after bleaching by light. The difference between the spectrophotometer readings is proportional to the amount of light-sensitive pigment in the extract. Resynthesis in the treated frogs was impaired.

Exposure to Light and Darkness	Rhodopsin per Frog	
	Treated	Controls
A. Dark 24 hr.	25	27
B. ,, 24 hr.; light 1 hr.	6	11
C. ,, 24 hr.; ,, 1 hr.; dark 1 hr.	12	42
D. ,, 26 hr.	37	57

Table II shows that exposure to light for only 1 hr. was effective in bleaching the greater part of the rhodopsin in the frog retina, and that a subsequent period of 1 hr. in the dark was sufficient for a considerable degree of resynthesis to take place. Frogs treated with 153C51 but kept in the dark retained most of their rhodopsin, and this was bleached in the normal manner when the animals were exposed to light (Table II). The drug prevented resynthesis when the animals were again put in the dark, and the effect was proportional to the dose (Fig. 4). Frogs which had been handled always gave higher rhodopsin figures

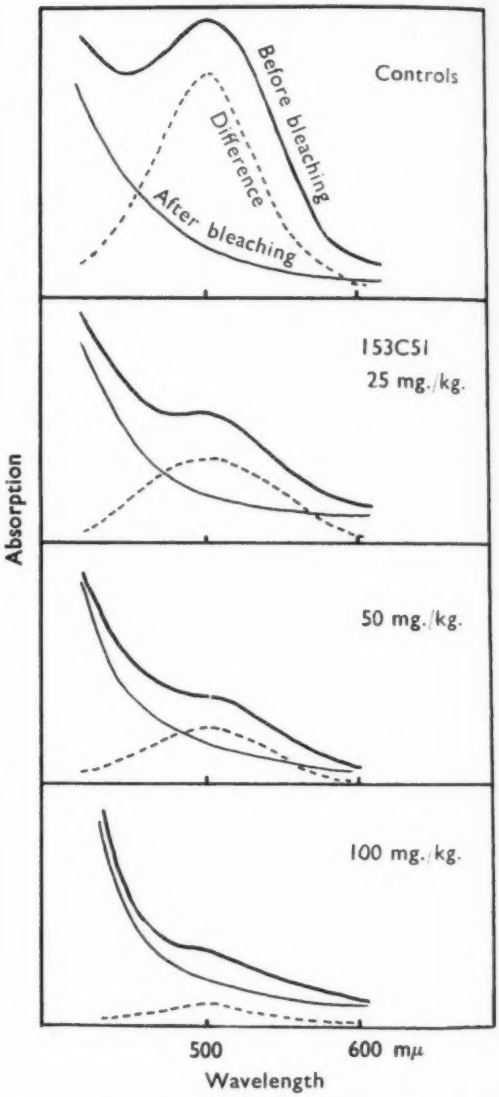


FIG. 4.—The effect of graded doses of 153C51 on the synthesis of rhodopsin in the frog retina. Groups of 3 frogs were used at each dose.

than frogs which had been left undisturbed; it was important therefore to give doses of frog-saline to control animals whenever doses of drugs were given to test groups. The dose-response curves obtained with the heptane derivatives are shown in Fig. 5. Doses given by mouth produced similar, but smaller, effects. The primary amines

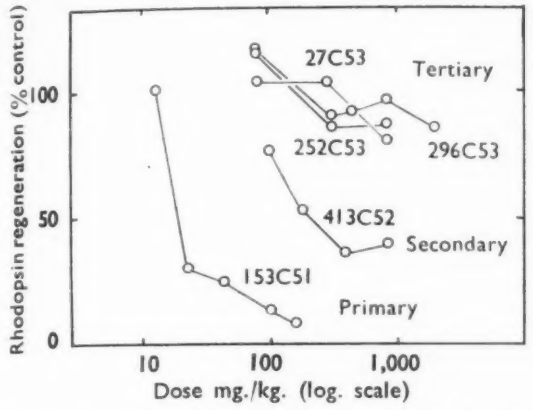


FIG. 5.—Dose response curves showing the relative toxicities of heptane derivatives to rhodopsin synthesis in the frog retina. Doses were given into the ventral lymph sac, and are calculated in terms of base. Each curve is the mean of several experiments.

were the most, and the tertiary the least, toxic. The effect of one course of 3 injections of 100 mg./kg. of 153C51 was sufficient to depress the rhodopsin extractable from the retinas of frogs exposed to standard light followed by dark conditions for 2 to 3 weeks. There was then a gradual return towards normal, but only 40% or less of normal ability to resynthesize rhodopsin was recovered 4 weeks after the drug was given. Some of the tertiary amines, such as 27C53, 296C53, and 252C53, had little or no action when given in the standard course of 3 doses. When large doses were given orally to frogs on 5 days a week for several weeks, the dimethyl-compound 27C53 produced toxic effects after 1 week and the diethyl-compound 296C53 showed an effect after 3 weeks (Fig. 6). The effect of the dihydroxyethyl-compound 252C53 was very slight.

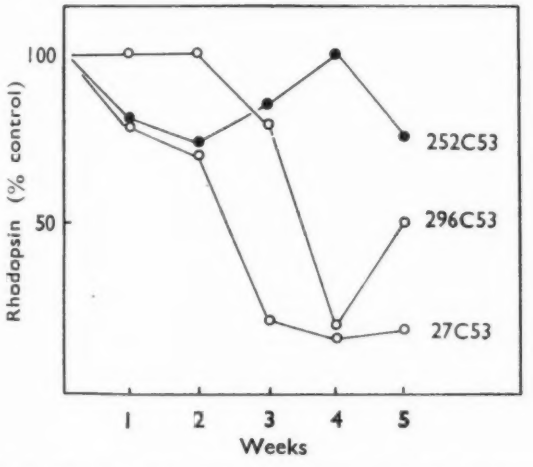


FIG. 6.—The effect of prolonged oral treatment with heptane derivatives on the rhodopsin content of frog retinas. Each drug was given daily for 5 days a week in doses of 200 mg./kg. Groups of 3 frogs were killed weekly and compared with a parallel group of frogs given 0.6% saline.

Large doses of natural or synthetic vitamin A given orally to frogs did not antagonize the retinotoxic effect of 153C51.

DISCUSSION

It is clear that an estimate of the toxicity of diphenoxyalkanes based upon intravenous LD50 values for mice would be misleading. From Table I it might appear that some of the primary amines are less toxic than secondary or tertiary derivatives. Nevertheless, the more important effect on the retina is most pronounced with primary amines. The effect on the retina is one of the most interesting aspects of the toxicity of diphenoxyalkanes. Carnivores and primates appear to be more sensitive than rodents to the retinotoxic action of oral doses of the drugs. In addition, it has been found by Dr. J. Newsome (private communication) that the tertiary amino-derivatives 296C53 and 252C53, which have low toxicity to the eye and powerful schistosomicidal activity in mice, have no detectable activity on *S. mansoni* infection in a baboon. These findings suggest that the drug itself, or secondary and primary amines derived from it, may be the retinotoxic agents, and that mice, guinea-pigs, and rabbits are capable of metabolizing diaminodiphenoxyalkanes given orally into substances which kill schistosomes but have minimal effects on the eye. A wide survey of the series has shown that retinotoxic activity in the frog and cat does not run parallel to schistosomicidal potency in the mouse, and it is possible that a member of the series will eventually be found which shows high schistosomicidal activity in primates without dangerous side-effects on the retina. Compounds which depart from the general structure in having amino-groups in the *m*- or *o*-, instead of the *p*-, position, and those with hydroxyl-groups substituted in the benzene rings, have neither retinotoxic nor schistosomicidal activity.

It is tempting to suggest that some of the toxic actions of the drugs may be brought about by interference with the biological activity of vitamin A. However, attempts to antagonize the

effects of the drugs on the hair and the retina with large doses of preparations containing synthetic or natural vitamin A met with no success. There is some evidence that the drugs are inhibitors of alcohol dehydrogenase, and this may be one way in which they could interfere with the synthesis of rhodopsin in the retina. Alcohol dehydrogenase catalyses the formation of the aldehyde retinene from vitamin A alcohol; the retinene then combines with retinal protein to form rhodopsin (Collins, 1954). An understanding of the mode of action of compounds of this series depends on a fuller investigation of their metabolism and of the effects of the drugs and their metabolites on biochemical processes.

We wish to acknowledge the permission of Dr. J. Newsome, of the Medical Research Council Bilharzia Unit, for permission to quote his results. We are grateful to Dr. H. J. A. Dartnall for advice on the extraction and measurement of rhodopsin and to Dr. W. F. J. Cuthbertson for a sample of vitamin A concentrate.

References

Caldwell, A. G., and Standen, O. D. (1956). *Brit. J. Pharmacol.*, **11**, 367.

Collins, F. D. (1954). *Biol. Rev.*, **29**, 453.

Collins, R. F., Davis, M., and Hill, J. (1954). *Chem. & Ind.*, **35**, 1072.

Edge, N. D., Mason, D. F. J., Wien, R., and Ashton, N. (1956). *Nature, Lond.*, **178**, 806.

Evelyn, K. A., and Malloy, H. J. (1938). *J. biol. Chem.*, **126**, 655.

Raison, C. G., and Standen, O. D. (1954). *Trans. R. Soc. trop. Med. Hyg.*, **48**, 446.

—— —— (1955). *Brit. J. Pharmacol.*, **10**, 191.

Standen, O. D., and Walls, L. P. (1956). Ibid., **11**, 375.

Zewi, M. (1941). *Acta physiol. scand.*, **1**, 271.

Brit. J. Pharmacol. (1957), **12**, 475.

THE EFFECT OF HUMAN PLASMA ON THE GLUCOSE UPTAKE OF THE RAT DIAPHRAGM BEFORE AND AFTER ADMINISTRATION OF CARBUTAMIDE

BY

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The glucose uptake of the rat diaphragm has been determined in the presence of the plasma of young volunteers before and after administration of carbutamide without and with added insulin. The increase in the glucose uptake of the rat diaphragm due to the added plasma above that in the medium alone has been termed plasma effect. The increase in the glucose uptake of the rat diaphragm with plasma and added insulin above that with only insulin in the medium has been termed plasma + insulin effect. There was a significant increase in the plasma effect and the plasma + insulin effect after carbutamide administration. The increase in the plasma + insulin effect was significantly greater than the increase in the plasma effect. From these observations it has been suggested that carbutamide potentiates the action of insulin peripherally. Observed facts about carbutamide do not contradict this mechanism of action.

Since the articles published in 1955 about the oral anti-diabetic drug, carbutamide (BZ-55), work has been carried out at various places to confirm its hypoglycaemic property and to study the mechanism of its action. The former action is now fairly well established and the drug has been recommended in certain types of diabetes mellitus.

The mechanism of the hypoglycaemic action is, however, uncertain. Originally from the histological studies of the pancreas, it was suggested that BZ-55 caused structural destruction (Frank and Fuchs, 1956) or functional inhibition of the α cells. Other observers failed to confirm this (Ferner and Runge, 1956). Mirsky, Perisutti, and Diengott (1956) suggested that BZ-55 inhibited the insulin destroying system (insulinase). Vaughan (1956) could not confirm this. She as well as others (Hawkins, Ashworth, and Haist, 1956; Tyberheim, Hasley, and Williams, 1956) showed that BZ-55 prevented glycogenolysis in the liver. Young (1956) introduced a novel idea by proposing that this and the other hypoglycaemic substances destroyed the organisms in the pool at the bottom of the portal drain which might be responsible for the destruction of insulin. But he himself has doubted this, as another hypoglycaemic compound, tolbutamide (D860, Orinase), is devoid of antibacterial activity. Loubatieres (1955), Ashworth and Haist (1956), and Pozza, Galansino, and Foa (1956) maintain that BZ-55 stimulates insulin secretion.

Gemmill (1940) first showed that insulin increases glycogen synthesis by the isolated rat diaphragm. Recently Randle (1954) and Vallance-Owen, Hurlock and Please (1955) have studied the effect of plasma on the glucose uptake of the rat diaphragm as a means of estimating plasma insulin levels. The experiments reported here are concerned with the effect of plasma on the glucose uptake of the isolated rat diaphragm before and after administration of BZ-55 and also with the effect of such plasma with added insulin.

METHODS AND MATERIAL

Ten housemen of Sassoon Hospital, Poona, aged 23 to 28 years, acted as subjects for this work. Each volunteer fasted from 10 p.m. on the previous night. Ten ml. of venous blood was collected in a heparinized syringe at 8 a.m. One ml. of this blood was used for blood sugar estimation, which was made by the Folin method. The remaining blood was centrifuged to separate the plasma, which was stored in a refrigerator but was always used within one hour of its withdrawal.

White rats, weighing between 120 to 180 g., were used. Each rat was starved for 24 hr., killed by stunning, and the two hemi-diaphragms removed quickly with as little trauma to the tissue as possible. Each hemi-diaphragm was bisected and each of the four quarter diaphragms was incubated in one of the following: (I) medium; (II) plasma-medium mixture; (III) plasma-medium-insulin mixture; (IV) insulin-medium mixture. The Stadie and Zapp (1947) phosphate buffer (*p*H 6.8) containing 0.2%

glucose was used as the medium. The plasma-medium mixture contained 2 ml. plasma + 2 ml. medium. The plasma-medium mixtures were prepared as directed by Randle (1954), but the proportions were different. The plasma-medium-insulin mixture was plasma-medium mixture containing 10^{-3} units of insulin/ml. The insulin-medium mixture was medium containing 10^{-3} units of insulin/ml. It was found by direct measurement that the glucose concentration in the plasma-medium mixture did not fall below 0.15%. The volume of incubation fluid was 2 ml. in each case.

Incubation was carried out in the Dubnoff metabolic shaking incubator at 38° with 100% oxygen as gas phase for a period of 90 min. with a shaking rate of 112 oscillations/min. Simultaneously, four similar vessels containing 2 ml. each of the above incubation fluids but without the diaphragms were incubated as controls. At the end of the incubation period the diaphragms were weighed, and the glucose in the control and the incubated samples estimated by the Folin micromethod. The difference between the control and the incubated samples was taken as the glucose uptake by the rat diaphragm, which was calculated as mg. glucose/g. wet weight of the diaphragm/hr. The difference between (II) and (I) was taken as the plasma effect (Table II) and the difference between (III) and (IV) was taken as the plasma+insulin effect. (I) and (II) did not contain any added insulin. Here the increase in (II) over (I) is due to the plasma when the insulin concentration in the medium was zero. (III) and (IV) contained insulin in the concentration of 10^{-3} units of insulin/ml. The increase in (IV) over (I) was due to the added insulin. This increase must also be present in (III). But (III) contains plasma in addition. Thus increase in (III) over (IV) is due to the plasma over the basal concentration of insulin, which was 10^{-3} units/ml. This was termed as plasma+insulin effect, by which is meant the effect of plasma alone in the presence of added insulin.

On the day the first blood sample was taken each volunteer received 1 g. of carbutamide (Invernol, Hoechst-Fedco Pharma) on two occasions. Thereafter, 0.5 g. was given three times a day on the subsequent three days ; 0.5 g. on the morning of the fourth day. A fasting sample of blood was collected 2 hr. later and treated in the same way as the previous one. Each volunteer thus received a total of 5.5 g. of BZ-55 in 68 hr.

Results

All the volunteers remarked on the increase in their appetite on the third and the fourth days. Most of them complained of dizziness and cramps in their legs. A few of them complained of hypotonia and mental excitement. Two complained of tremors and unsteadiness on the morning of the fourth day. Symptoms were aggravated when meals were delayed and relieved after taking food. The symptoms persisted for a day after withdrawal of the drug and in one case for two days. Column (c) of Table I gives the fall in the fasting blood sugar after taking the drug. The mean of the fall was 42.7 mg.%. None of the volunteers developed hypoglycaemic shock.

TABLE I

FASTING BLOOD SUGAR OF NORMAL MEN BEFORE AND AFTER ADMINISTRATION OF BZ-55

No. of Man	Fasting Blood Sugar (mg.%)		
	Before BZ-55 (a)	After BZ-55 (b)	Fall after BZ-55 (c)=(a)−(b)
1	106	72	34
2	98	66	32
3	90	48	42
4	100	41	59
5	114	38	76
6	105	64	41
7	84	56	28
8	90	60	30
9	102	66	36
10	107	58	49
			Mean 42·7

In Table II, cols. (a) and (b) give the plasma effect before and after administration of BZ-55 respectively. Column (c) gives the increase in the plasma effect ((b)−(a)) after BZ-55 administration. Columns (d) and (e) give the plasma+insulin effect before and after administration of BZ-55 respectively, and col. (f) gives the increase in it ((e)−(d)) after BZ-55.

The mean plasma effect and the mean plasma+insulin effect before BZ-55 administration were 0.689 and 0.639 respectively with practically no difference between the two. After BZ-55 administration the mean values of these effects were 1.130

TABLE II

PLASMA EFFECT AND PLASMA+INSULIN EFFECT BEFORE AND AFTER ADMINISTRATION OF CARBUTAMIDE (BZ-55)

Tissue, isolated rat diaphragm muscle. Plasma effect=Glucose uptake in [plasma+medium]−glucose uptake in the medium. Plasma+insulin effect=Glucose uptake in [plasma+medium+insulin]−glucose uptake in [medium+insulin]. Insulin concentration 10^{-3} units/ml. in each experiment. Medium=The Stadie and Zapp (1947) phosphate buffer (*p*H 6.8) containing 0.2% glucose. For further explanation see text.

No. of Man	Plasma Effect (mg./g./hr.)			Plasma+Insulin Effect (mg./g./hr.)		
	Before BZ-55 (a)	After BZ-55 (b)	Increase After BZ-55 (c)	Before BZ-55 (d)	After BZ-55 (e)	Increase After BZ-55 (f)
1	0·20	1·20	1·00	0·20	2·80	2·60
2	1·10	0·56	−0·54	0·40	1·20	0·80
3	0·80	1·80	1·00	0·20	1·50	1·30
4	1·29	2·27	0·98	0·80	0·32	−0·48
5	0·40	0·52	0·12	0·05	2·05	2·00
6	0·75	0·94	0·19	1·23	2·56	1·33
7	0·70	1·16	0·46	1·16	1·86	0·70
8	0·86	1·53	0·67	1·30	2·20	0·90
9	0·33	0·42	0·09	0·45	1·28	0·83
10	0·46	0·90	0·44	0·60	2·28	1·68
Mean	0·689	1·130	0·441	0·639	1·805	1·166

and 1.805 respectively. The plasma + insulin effect is significantly greater than plasma effect after BZ-55 administration ($t=2.24$, $P<0.05$). The absence of any difference between the two values before BZ-55 administration suggests that the starting point on the dose/response curve does not significantly affect the increase in the glucose uptake due to the plasma. Hence the greater increase in the plasma + insulin effect seen after BZ-55 administration is unlikely to be due to this factor. The mean increase in the plasma effect after BZ-55 administration was 0.441. The mean increase in the plasma + insulin effect after BZ-55 administration was 1.166, which is significantly greater than the increase in the plasma effect ($t=2.34$, $P<0.05$). Our results thus show a fall in the fasting blood sugar, an increase in the plasma effect and an increase in the plasma + insulin effect. The increase in the plasma + insulin effect is significantly greater than the increase in the plasma effect.

Discussion

Field and Woodson (1956) have reported that the increase in glycogen synthesis of rat diaphragm due to insulin was depressed by BZ-55 and sulphadiazine in concentrations of 2.0 mg./ml. The insulin effect was not affected by BZ-55 in a concentration of 0.2 mg./ml. Greater concentrations of BZ-55 without insulin did not affect glycogen synthesis of the rat diaphragm. Cahill, Hastings, and Ashmore (1957) have reported that BZ-55 and tolbutamide did not alter the insulin effect on glucose uptake of the rat diaphragm in a concentration of 50 mg.% with insulin concentrations ranging between 0.25 milliunits to 25 milliunits/ml. Clarke, Davidson, Schonbaum and Senman (1956) reported that BZ-55 in a concentration of 6.0 mg.% caused depression of glucose uptake of the rat diaphragm. Addition of insulin overcame this depression and produced the usual increase in the glucose uptake, thus giving an apparent increase in the insulin effect. Though the authors do not think that this was due to increased glucose uptake in presence of BZ-55 + insulin, they do not rule out such a possibility.

We used plasma containing BZ-55 to test the effect on the glucose uptake of the rat diaphragm. Hence the drug was present in the form in which it circulates in the plasma and in the concentration in which it caused hypoglycaemia in the volunteers. From the results presented in the Tables it is obvious that the insulin-like effect of the plasma increased after the drug administration. Our finding that the increase in the plasma + insulin effect was significantly greater than the increase in the plasma effect is perhaps more important. There was practically no difference between the plasma effect and the plasma + insulin effect before administration of BZ-55. After BZ-55 administration the plasma contained free sulphonamide; the plasma also contained insulin; and an increase in the plasma effect after BZ-55 administration might be explained by assuming that BZ-55 in the plasma enhanced the action of whatever insulin was present there. In the case of plasma + insulin effect there was a greater amount of insulin in the medium and hence the increase in the plasma + insulin effect is greater than the increase in the plasma effect. Hence our results suggest that BZ-55 potentiates the peripheral action of insulin.

If the increase in the plasma effect and the increase in the plasma + insulin effect were due to the direct action of the drug present in the plasma, then the increase in the plasma effect should not have been different from the increase in the plasma + insulin effect, because the same amount of the drug was added to test both effects, namely that present in 1 ml. of plasma. On the contrary, we obtained a significantly greater increase in the plasma + insulin effect.

Similarly if we assume that BZ-55 increases the insulin concentration of the plasma by either stimulating its secretion (Ashworth and Haist, 1956; Loubatieres, 1955) or preventing its destruction (Mirsky, 1956), then we would not have expected to obtain the statistically significant difference between the plasma effect and the plasma + insulin effect after administration of BZ-55 which we found (Table II, cols. b and e).

Our assumption that BZ-55 potentiates the peripheral action of insulin is not incompatible with the observed facts about BZ-55 action.

It has been observed that BZ-55 lowers the blood sugar to a certain level. In rabbits if the dose is still further increased hyperglycaemia results (Achelis and Hardebeck, 1956). When a man commences taking the drug, it will cause a fall in the blood sugar as it will enhance the action of insulin that is present in the plasma. As the blood sugar falls the insulin secretion will decrease. This decrease will progress until, at a certain degree of hypoglycaemia, insulin release ceases and no further fall in the blood sugar will occur. As the insulin disappears from the plasma, there will be rapid glycogenolysis and hyperglycaemia will result.

Failure of the drug in diabetes mellitus cases with very high blood sugar is to be expected

because in these cases very little insulin, if any, is present in the plasma (Vallance-Owen *et al.*, 1955). It then follows that BZ-55 should be most effective in cases with relative insulin deficiency. It is an accepted fact that cases suitable for BZ-55 therapy are those of obese, mild diabetics at or over middle age. When a patient receives insulin regularly over a prolonged period of time, it is possible that secretion of insulin in such a patient is depressed as happens with most of the other hormones. This would explain the failure of BZ-55 in cases which have received prolonged insulin therapy. Our view is strengthened by the observation that BZ-55 when given alone does not cause hypoglycaemic shock, but, after its administration with smaller doses of insulin, hypoglycaemic shock may arise (Bertram, Benfeldt, and Otto, 1956).

The tendency of BZ-55 to cumulate should be borne in mind in its clinical use. Lowering of the blood sugar below the physiological limits may convert a relative insulin deficiency into an absolute one by prolonged depression of β cells. This can be avoided by reducing the maintenance dose of the drug to the minimum and ensuring that the fasting blood sugar does not fall below normal limits.

References

Achelis, J. D., and Hardebeck, K. (1956). *German med. J.*, **1**, 5.

Ashworth, N. A., and Haist, R. E. (1956). *Canad. med. Ass. J.*, **74**, 956.

Bertram, F., Benfeldt, E., and Otto, H. (1956). *German med. J.*, **1**, 8.

Cahill, G. F., Hastings, A. B., and Ashmore, J. (1957). *Diabetes*, **6**, 26.

Clarke, D. W., Davidson, M., Schonbaum, E., and Senman, H. (1956). *Canad. med. Ass. J.*, **74**, 966.

Ferner, H., and Runge, W. (1956). *German med. J.*, 1, 50.

Field, J. B., and Woodson, M. L. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 534.

Frank, F., and Fuchs, J. (1956). *German med. J.*, **1**, 1.

Gemmill, C. L. (1940). *Johns Hopk. Hosp. Bull.*, **66**, 232.

Hawkins, R. D., Ashworth, N. A., and Haist, R. E. (1956). *Canad. med. Ass. J.*, **74**, 972.

Loubatieres, A. (1955). *Pr. méd.*, **63**, 1701.

Mirsky, I. A., Perisutti, G., and Diengott, D. (1956). *Metabolism*, **5**, 156.

Pozza, G., Galansino, G., and Foa, P. P. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 539.

Randle, P. J. (1954). *Brit. med. J.*, **1**, 1237.

Stadie, W. C., and Zapp, J. A. (1947). *J. biol. Chem.*, **170**, 55.

Tyberheim, J. M., Hasley, T. D., and Williams, R. H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 322.

Vallance-Owen, J., Hurlock, B., and Please, N. W. (1955). *Lancet*, **2**, 583.

Vaughan, M. (1956). *Science*, **123**, 885.

Young, F. G. (1956). *Brit. med. J.*, **2**, 431.

Brit. J. Pharmacol. (1957), **12**, 479.

THE EFFECT OF METHYLPENTYNOL IN MAN

BY

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Administration of methylpentynol to human subjects depressed their autonomic reactions to a difficult motor task and impaired their performance. Hexobarbitone affected neither autonomic reactions nor performance, though it impaired hand steadiness. The results suggest that the modes of action of methylpentynol and of hexobarbitone are different.

Methylpentynol has been shown to have a sedative action in animals (Margolin, Perlman, Villani and McGavack, 1951), and appears to reduce fear in rats (Dicker, Steinberg, and Watson, 1957). In human subjects the drug has been used to allay anxiety (Trotter, 1953 ; Bourne, 1954), without adversely affecting performance of motor tasks (Trotter, 1954). Furthermore, methylpentynol blocks transmission in autonomic ganglia (Quilliam, 1957).

As is well known, performance of difficult tasks can affect autonomic functions, indicated for example by pulse rate, blood flow, as well as respiration rate. Our purpose was to investigate whether methylpentynol would, first, alter such autonomic reactions to difficult tasks, secondly, affect performance in these tasks, and, thirdly, be distinguishable from a barbiturate in these respects.

METHODS

The subjects were 20 male first-year medical students (average age 20) who were unfamiliar with the psychological and physiological techniques used. Each subject was seen once only and was given by mouth a drug or a control 25 min. before experiment. Drugs were dissolved in the same solvent ; the control consisted of the solvent alone. Eight subjects were seen between 12 and 1 p.m., the rest between 4 and 5 p.m., that is at least 3 hr. after a meal. Drugs and control were allocated at random and the subjects were not told which they received.

The subjects sat in an adjustable chair, and apparatus for recording pulse rate, blood flow and respiration was attached. Continuous recordings of these were taken ; skin temperature was measured at 60 sec. intervals. To measure respiration, an inflated rubber bag tied round the thorax of the subject was connected to a tambour and so to a pen recorder. Blood flow was measured by venous occlusion plethysmography (Lewis and Grant, 1925 ; Barcroft and Swan, 1953) from the left arm and skin temperature with a thermocouple (Paton and Steinberg, 1956) on the forehead.

The method for recording pulse rate was as follows: a small lever connected to a tambour (diameter 3 cm.) was fixed on the wrist over the radial artery. The oscillations of the lever were transmitted to a smaller tambour (diameter 8 mm.) supporting a mirror which reflected a light beam to a photo-transistor. Illumination of the photo-transistor produces a small electrical pulse which, amplified and converted into a pulse of a pre-set width (Fig. 1), is recorded by means of a pen on an Ediswan oscillograph. The electrical pulse activates a relay which short-circuits the input of the power amplifier, causing the pen to return to the base line. At the end of the pulse the relay opens, allowing the pen recorder to move again at a speed determined by the value of the time constant of C and R (Fig. 1). This movement continues until the arrival of the next pulse which terminates the discharge of C and brings the pen to base line. The amount of discharge is therefore proportional to the interval between successive pulses, that is to the pulse rate. This means, as can be seen from Fig. 4, that the faster the pulse beats, the smaller the amplitude of the tracing and conversely.

While these recordings were being made, the subjects were asked to carry out two psychological tests: a test of complex motor co-ordination and a hand steadiness test. The tests were given to different subjects in alternate orders. The whole experiment lasted about one hour.

The apparatus to test motor co-ordination was a modification of the Craik Triple Tester: it consists of a rotating horizontal drum round which is marked a spiral path of irregularly placed dots. The subject steers a wheel which guides a pointer on the drum. He is asked to drive the pointer over as many dots as possible, each "hit" being scored on an electric counter. The apparatus was used to assess the subject's performance and his "level of aspiration" (Eysenck and Himmelweit, 1946). Performance was the number of hits/trial of 45 sec. After each trial the subject was told his score and was asked to

predict his performance in the next trial; the difference between score in the previous trial and prediction of the next performance was taken as the "level of aspiration." The subject made six trials separated by 1 min. intervals (see Steinberg, 1954).

In the hand steadiness test, the subject was asked to hold for two periods of 30 sec. a stylus (1.57 mm. diameter) in a hole of 3.175 mm. diameter without touching its side. The apparatus, a modification of the Whipple steadiness test (1914), is built in such a way that any contact of the stylus against the side of the hole is recorded on an electric counter at a rate of 20/sec. Hence the longer the contact the higher the score.

Drugs.—Methylpentynol was used in the form of "Oblivon" elixir (British Schering). The dose administered was 500 mg. in 8 ml. (Trotter, 1954). Hexobarbitone sodium (*B.P.*) was dissolved in the same solvent as methylpentynol, dose 150 mg. in 8 ml. This dose was chosen as the maximum which did not produce drowsiness. The solvent used for both hexobarbitone and control was flavoured to resemble "Oblivon" elixir and was supplied by the makers.

Statistics.—Changes in pulse rate and blood flow were expressed in percentages. Means are given with their standard errors. Chi-square and "Student's" *t* tests have been used. The Mann-Whitney U test (Mann and Whitney, 1947) was applied to results of psychological tests where the distributions were skewed.

Results

Autonomic Reactions

Preliminary Resting Period.—At the beginning of the experiment, the subjects were asked to relax. All appeared restful and quiet, though the mean pulse rate of controls was higher than that of subjects with methylpentynol or hexobarbitone. During the period of 3 to 5 min. before the first psychological test, the mean pulse rate of controls was 76 ± 1.2/min. (7), that of subjects with methylpentynol 70 ± 1.2/min. (7), and with hexobarbitone 67 ± 2.5/min. (6). The differences between the control group and the groups with either drug were statistically significant, $t = 3.141$, $P < 0.01 > 0.001$ and $t = 2.639$, $P < 0.05 > 0.02$, respectively. Mean skin temperature was similar in the three groups: control $32.7 \pm 0.33°$, methylpentynol $31.1 \pm 0.39°$, and hexobarbitone $32.9 \pm 0.35°$. The tracings showed no difference in the rate of respiration.

Experimental Period.—While the tests were being explained both pulse rate and blood flow increased in control subjects. This is shown for the Triple Tester (Figs. 2 and 3).

During the actual performance of tests, pulse rate and blood flow increased markedly in controls and in subjects with hexobarbitone; the increases were especially striking during performances with the Triple Tester. By contrast, in subjects with methylpentynol pulse rate and blood flow remained practically unchanged (Figs. 2 and 3). During tests the respiration of controls became faster and shallower; in subjects with methylpentynol the respiration became markedly deeper, though its rate did not change noticeably (Fig. 4). Hexobarbitone did not seem to affect respiration. Skin temperature did not change appreciably in any group.

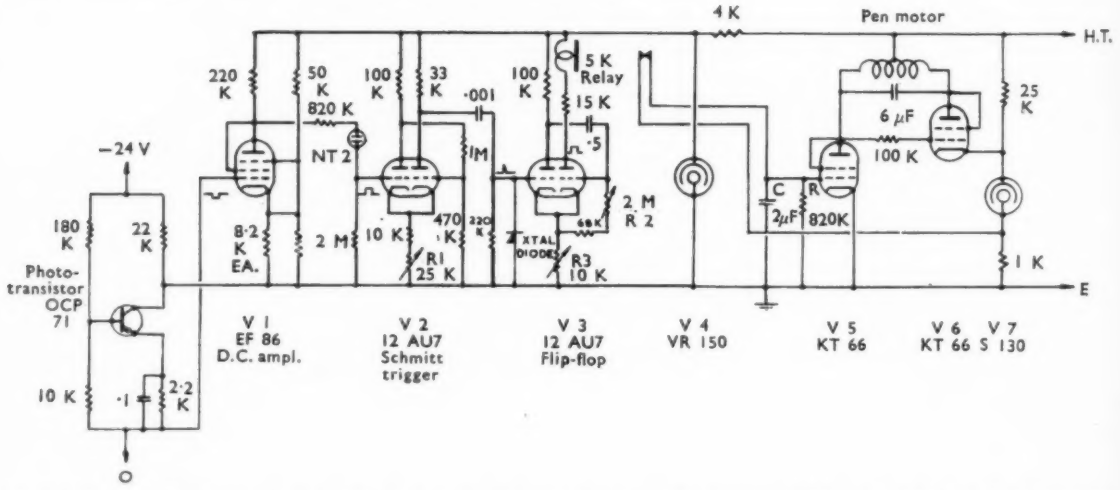

FIG. 1.—Diagram of the electronic circuit for the pulse rate recording device. When excited by a beam of light, the photo-transistor develops a negative voltage, which, amplified by V_1, is fed to a Schmidt trigger, V_2, built with a pre-set control, R_1. The output from the Schmidt trigger operates a "flip-flop," V_3, which produces a pulse; the width of the latter is pre-set by R_2. The purpose of V_3 is to ensure that the relay remains closed for sufficient time to allow the pen to return to base line. The voltage produced in the C/R network is amplified and fed to the pen recorder by means of a pair of power valves, V_5 and V_6.

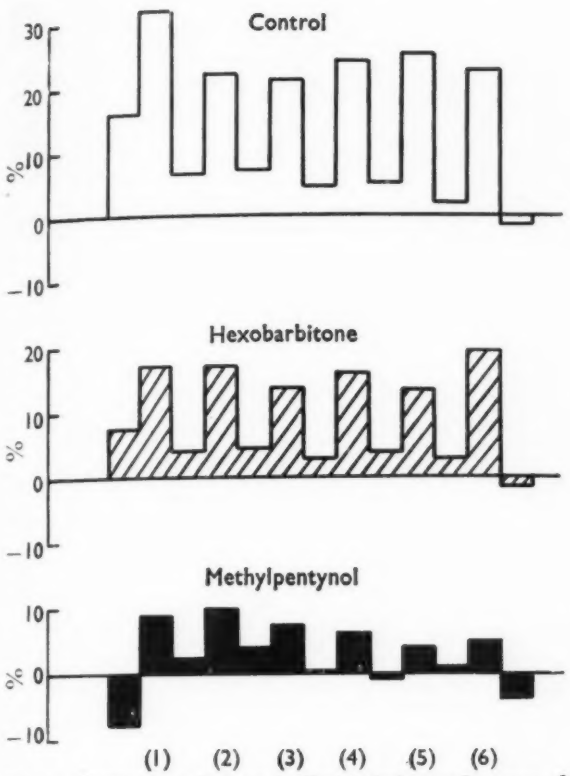

FIG. 2.—Changes in pulse rate (ordinate) during performance of Triple Tester, in control subjects and in those who had received hexobarbitone (150 mg.) or methylpentynol (500 mg.). The changes are expressed as % of resting levels. The numerals in parentheses show the order of the series of trials.

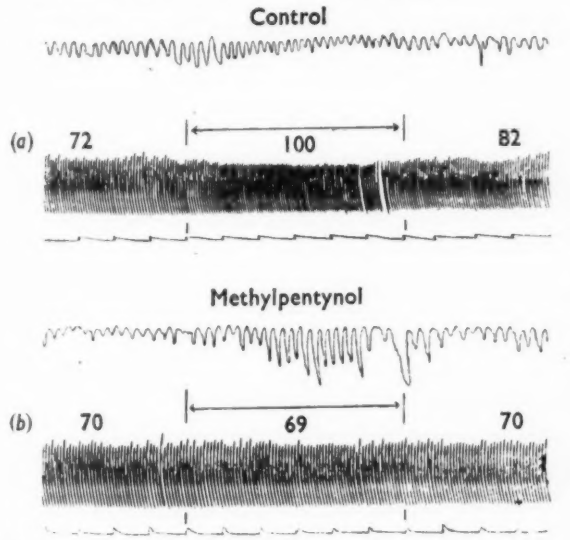

FIG. 4.—Respiration and pulse rate of (*a*) control subject and (*b*) a subject who had received methylpentynol before, during, and after a difficult test was performed. The test was carried out during the period between the vertical lines on each record. Upper trace, respiration; middle trace, pulse rate; lowest trace, time, 10 sec. The numerals give the pulse rate/min.

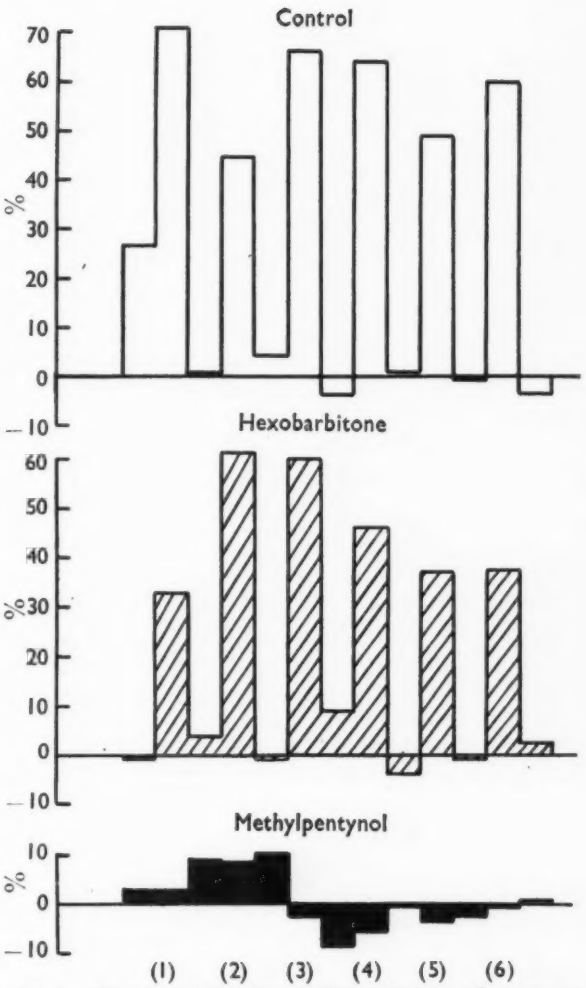

FIG. 3.—Changes in blood flow (ordinate) during performance on Triple Tester in control subjects and in those who had received hexobarbitone (150 mg.) or methylpentynol (500 mg.). Changes are expressed as % of resting level. The numerals in parentheses show the order of the series of trials.

Test Performances

Triple Tester.—Performance improved with each trial in all three groups. The actual scores were, however, significantly lower in subjects with methylpentynol than in controls, $P=0.036$ (Fig. 5). Subjects with methylpentynol also had a lower "level of aspiration" than controls ($P=0.019$). Hexobarbitone did not affect performance or "level of aspiration" (Fig. 6).

Steadiness Test.—The performance of subjects with methylpentynol was similar to that of controls. The mean scores during 60 sec. were 195 ± 53.3 (6) and 193 ± 74.7 (5) respectively. Subjects with hexobarbitone, however, were nearly twice as unsteady. Their mean score was 377 ± 33.6 (6), ($t=2.390$, $P<0.05>0.02$).

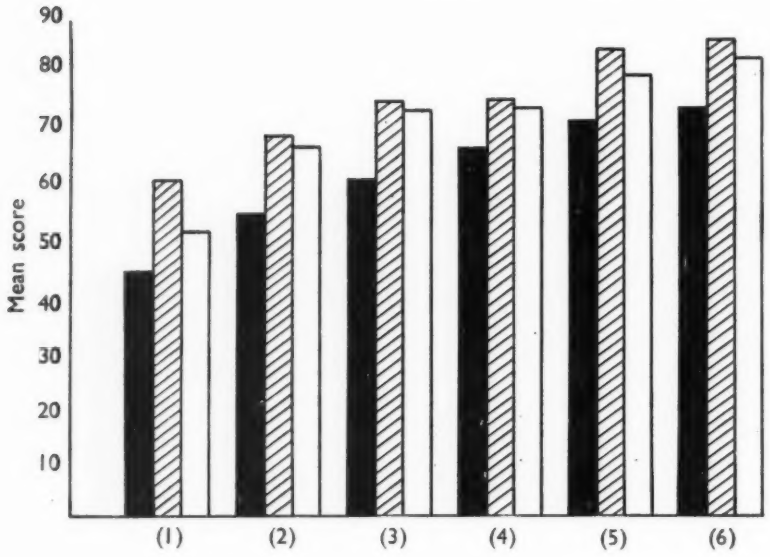

FIG. 5.—Performance on Triple Tester. Ordinates are the mean scores in 6 trials. Open columns, controls; cross-hatched columns, hexobarbitone (150 mg.); solid columns, methylpentynol (500 mg.). The numerals in parentheses show the order of the series of trials.

Subjects' Sensations

At the end of the experiment subjects were questioned about their sensations, and their answers were classified as (a) "tense," (b) "relaxed," (c) "drowsy" and (d) "normal." Table I shows that most subjects with methylpentynol felt "relaxed," while most controls felt "tense" ($P=0.04$).

TABLE I
DISTRIBUTION OF SUBJECTS' SENSATIONS

	Methylpentynol	Control	Hexobarbitone
Tense ..	1	5	0
Relaxed ..	5	1	3
Drowsy ..	1	2	3
Normal ..	1	1	3

DISCUSSION

On the assumption that increases in pulse rate and blood flow are reactions to the situation created by the performance or anticipation of difficult tests, it seems that methylpentynol is effective in that it decreases these autonomic responses. Subjects with methylpentynol, however, performed worse with the Triple Tester than controls and their "level of aspiration" was also lower; their prediction, however, was not more accurate. This suggests that, unlike controls, they were indifferent about their performance. This raises the question of whether people faced with a difficult task benefit from the damping down of their autonomic reactions by methylpentynol. According to experiments reviewed by Farber (1955) anxiety may sometimes help performance. It may

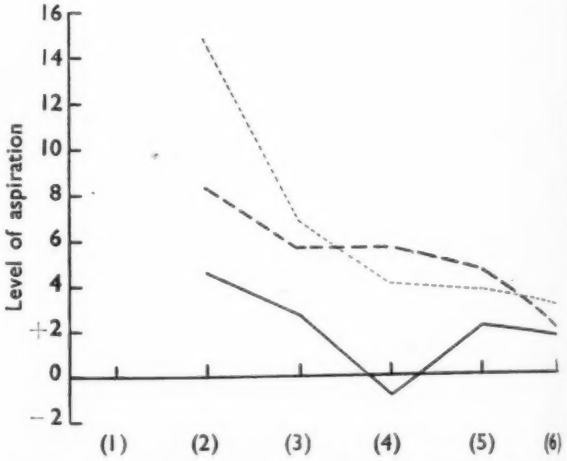

FIG. 6.—"Level of aspiration": difference between performance and prediction (ordinate). It will be noted that only 5 predictions could be made after the first trial. Full line, methylpentynol; dotted line, hexobarbitone; broken line, control. The numerals in parentheses show the order of the series of trials.

be noted that both the performance and the "level of aspiration" of subjects with hexobarbitone were similar to those of the controls, though their autonomic reactions remained fully responsive. This does not seem to be entirely attributable to the relatively small dose of hexobarbitone administered, as, during the resting period, the pulse rate of subjects with hexobarbitone remained lower and during the hand steadiness test their performance was worse than that of controls.

It would appear from the present investigation that the effect of methylpentynol on human sub-

jects faced with a difficult task is to make them perform worse and mind less.

We are grateful to British Schering Ltd. for generous supplies of "Oblivon" elixir, and to Miss Joan Nunn, Mr. P. E. Taylor, Mr. F. B. Ives and Mr. F. Ballhatchet for technical assistance. We want to express our thanks to Mr. G. L. Read, who designed and made the electronic apparatus for recording the pulse.

References

Barcroft, H., and Swan, H. J. C. (1953). *Sympathetic Control of Human Blood Vessels.* London: Edward Arnold.

Bourne, G. (1954). *Lancet*, **2**, 522.

Dicker, S. E., Steinberg, H., and Watson, R. H. J. (1957). *J. Physiol.*, **137**, 88P.

Eysenck, H. J., and Himmelweit, H. T. (1946). *J. gen. Psychol.*, **35**, 59.

Farber, I. E. (1955). *Psychol. Bull.*, **52**, 311.

Lewis, T., and Grant, R. T. (1925). *Heart*, **12**, 73.

Mann, H. B., and Whitney, D. R. (1947). *Ann. Math. Statist.*, **18**, 52.

Margolin, S., Perlman, P., Villani, F., and McGavack, T. H. (1951). *Science*, **114**, 384.

Paton, W. D. M., and Steinberg, H. (1956). *Brit. med. J.*, **2**, 622.

Quilliam, J. P. (1957). *Med. Press*, **138**, 121.

Steinberg, H. (1954). *Quart. J. exp. Psychol.*, **6**, 170.

Trotter, P. A. (1953). *Dental Practitioner*, **3**, 376.

—— (1954). *Lancet*, **2**, 1302.

Whipple, G. M. (1914). *Manual of Mental and Physical Tests.* Baltimore: Warwick and York.

Brit. J. Pharmacol. (1957), **12**, 484.

THE ANTIDIURETIC ACTION OF 5-HYDROXYTRYPTAMINE IN CATS IN RELATION TO THE PRODUCTION OF CERTAIN CHEMOREFLEXES

BY

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(RECEIVED MAY 29, 1957)

In cats lightly anaesthetized with cnloralose, small (5 to 50 μg./kg.) intravenous doses of 5-hydroxytryptamine temporarily reduced the flow of urine into the bladder in addition to causing reflex falls of blood pressure and heart rate and temporary arrest of breathing. Doses of phenyl diguanide and other aryl diguanides, the reflex effects of which on blood pressure and respiratory movement approximately matched those of 5-hydroxytryptamine, had comparatively little antidiuretic effect. Phenyl diguanide, unlike 5-hydroxytryptamine (tested previously under similar conditions), did not have an antidiuretic effect in hydrated mice in doses of 0.05, 1, and 2.5 mg./kg. subcutaneously. It is concluded that the reflex depressor action of 5-hydroxytryptamine plays little part in reducing urinary output.

Several different views have been expressed about the mechanism of the antidiuretic action of 5-hydroxytryptamine (5-HT). Corcoran, Masson, del Greco, and Page (1954) suggested that the antidiuretic effects observed in rats and dogs are, for the most part, brought about indirectly by stimulation of the posterior pituitary due to the pain of the injection and by lowering of blood pressure. Actually, antidiuretic doses of 5-HT in dogs have usually been found to raise rather than lower blood pressure (Abrahams and Pickford, 1956a and b ; Spinnazola and Sherrod, 1955). Del Greco, Masson, and Corcoran (1956) have since modified their views: they have observed that the antidiuretic effect of 5-HT in the rat is dependent on decreased glomerular filtration but largely independent of changes in blood pressure. Although Sala and Castegnaro (1953) attribute the antidiuretic action of 5-HT in the dog principally to an increased reabsorption of water from the renal tubules, other workers (Erspamer, 1954 ; Spinnazola and Sherrod, 1955 ; Abrahams and Pickford, 1956a) have found the antidiuretic effect of 5-HT to be attended by changes in glomerular filtration rate rather than in renal plasma flow. It has been shown that renal blood vessels are constricted by 5-HT (Page, 1952 ; Cerletti, Carpi, and Rothlin, 1955) and that the intact kidney pales when 5-HT is given intravenously (Erspamer, 1954 ; Abrahams and Pickford, 1956a). According to Erspamer and Asero (1952), 5-HT has a specific constrictor action on the afferent bed of the glomerulus.

Abrahams and Pickford (1956a) agree with Erspamer that vasoconstriction is a prerequisite for antidiuresis, but they believe that kidney blood vessels are no more sensitive to 5-HT than those of certain other areas. They noticed that when antidiuresis occurred after 5-HT had been given to dogs it was accompanied by obvious respiratory reactions. This was one of several pieces of evidence which suggested to them " that 5-HT produces antidiuresis mainly by setting up reflexes from the thorax, probably from the pulmonary bed, which, by altering the general blood pressure, affect the kidney."

Since there is a great difference between the cat and the dog as regards the effects of 5-HT on the circulatory and respiratory systems, we thought it of interest to see whether there is any obvious relationship between the antidiuretic activity of 5-HT in the cat and its " reflex " activity in that species. Whereas in the dog the coronary and allied chemoreflexes play little part in the circulatory response, in the lightly anaesthetized cat the chief circulatory effect of 5-HT is an abrupt fall of blood pressure and heart rate brought about reflexly by stimulation of chemoreceptors in the heart and lungs. In the dog 5-HT causes hyperpnoea by stimulating the aortic and carotid bodies,

but in the cat 5-HT causes temporary arrest of breathing by a chemoreflex involving sensory receptors in the lungs (Page, 1953 ; Dawes and Comroe, 1954 ; Kottegoda and Mott, 1955). We wondered whether the antidiuretic activity of 5-HT in the cat would be comparable to that in the dog despite these other differences. As it seemed possible that the hypotension evoked reflexly by 5-HT in the cat might contribute substantially to its antidiuretic action, we have tested in addition to 5-HT such compounds as phenyl diguanide which closely resemble 5-HT in their ability to produce the coronary and certain other chemoreflexes though not in most other respects (Dawes and Mott, 1950 ; Dawes and Comroe, 1954).

METHODS

Cats were anaesthetized with chloralose (usually 70 mg./kg.). The trachea and a saphenous vein were cannulated. Blood pressure was recorded from a carotid artery. Respiratory movements were recorded by a modification of the method of Gaddum (1941). To measure urine flow, the bladder was cannulated after the urethra had been tied and the cannula was connected by a long 2 mm. bore rubber tube to a Condon drop counter, which was placed on the ground 3 ft. below the level of the cat so that there was a slight negative pressure to prevent the urine from remaining in and distending the bladder.

Rabbits were anaesthetized with sodium pentobarbitone. Drug solutions were injected into them by a jugular vein.

The effect of phenyl diguanide on water diuresis in mice was studied by the technique of Waal and Veale (1956) which had been used in earlier experiments with 5-HT. During a 19 hr. period each mouse was given by stomach tube three doses of lukewarm water, each of 5% of its body-weight. Immediately after the third dose the mice were given a subcutaneous injection of 0.01 ml./g. of phenyl diguanide hydrochloride in 0.9% NaCl or of saline alone. To minimize individual variation, which was considerable, comparisons were made of the urinary output of groups of 4 mice. Each group was confined in a small cage above a collecting funnel and measuring cylinder. The amount of urine excreted was measured at 15 min. intervals for 6 hr. A water diuresis was produced in duplicate experiments, each with 8 batches of 4 mice tested on 4 different days. The mice of each batch eventually received 1 control injection of saline and 3 injections of phenyl diguanide hydrochloride, the test solutions of the latter containing 0.005, 0.1, and 0.25 mg./ml. respectively. To eliminate any effect due to tolerance, the treatments were randomized by two independent 4 by 4 latin squares.

The 5-HT used was serotonin creatinine sulphate monohydrate (May and Baker). The aryl diguanides were synthesized in the department.

RESULTS

Experiments on Cats.—Small doses of 5-HT (20 to 50 μg./kg.) decreased urine flow for periods ranging from 30 to 100 sec. in each of 10 female cats weighing 2.5 to 3.2 kg. Definite antidiuretic effects were produced in several of the cats with doses as low as 3 μg./kg. of the salt (almost equal to 1.5 μg./kg. of the base). The antidiuretic effect was often followed by an equally brief diuretic effect (Fig. 1). Long-lasting antidiuretic effects were not obtained even when 30 μg./kg. doses of 5-HT were given every 5 min. for 30 to 60 min. The duration of the antidiuresis obtained with a 30 μg./kg. dose appeared to depend on the initial urine flow ; the greater the rate of flow of urine the more transient was the antidiuretic effect.

In the same range of doses, 5-HT usually caused a temporary fall of blood pressure and arrest of breathing (Fig. 1). The antidiuretic effect

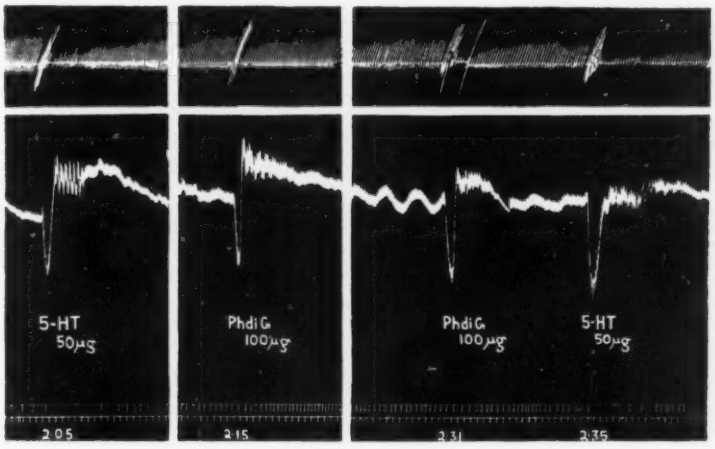

FIG. 1.—Cat. 3.2 kg. female. 210 mg. chloralose. Records from above downwards: respiratory movement, arterial blood pressure, urinary output, time (10 sec.). Note that phenyl diguanide (PhdiG) had comparatively little antidiuretic effect in amounts which had nearly the same effect on blood pressure and respiratory movement as the doses of 5-hydroxytryptamine (5-HT) used.

occurred after the trough of the hypotension. Several lines of evidence indicated that the antidiuretic effect is largely independent of the reflex actions on the circulatory and respiratory systems.

Firstly, with 3 of the cats pressor responses to 5-HT were obtained for a period of the experiment. Despite this reversal (for no ascertainable reason) of the normal effect of 5-HT on blood pressure, an antidiuretic effect was still produced (Fig. 2). Antidiuretic effects were sometimes obtained also in the absence of the respiratory reflex. Conversely, the usual reflex effects of 5-HT were obtained in a few instances in the absence of an antidiuretic effect.

Secondly, the reflex effects of 5-HT on blood pressure and respiratory movement could be matched fairly closely by injecting phenyl diguanide in suitable doses (Fig. 1). The 5-HT was about twice as active as phenyl diguanide on a molar dosage basis. In experiments performed on 8 of the cats, the antidiuretic effect of 5-HT was invariably greater than that of phenyl diguanide given in equidepressor amounts. In fact, doses of phenyl diguanide sufficient to lower blood pressure by 50 mm. Hg or more often had little or no antidiuretic effect. A brief diuretic response to phenyl diguanide was noted sometimes, especially when there was a secondary rise of blood pressure after the hypotension. With the *o*-, *m*-, and *p*-chlorophenyl diguanides and the *o*-, *m*-, and *p*-tolyl diguanides, as with phenyl diguanide, the antidiuretic effects (if any) were substantially less than those produced by 5-HT in equidepressor doses. In one experiment, acetylcholine and amyl nitrite were given in doses which lowered blood pressure to approximately the same extent as large doses of 5-HT and phenyl diguanide. All 4 drugs had a transient antidiuretic effect (Fig. 3). The antidiuretic effects of acetylcholine and amyl nitrite were comparable with those of phenyl diguanide, possibly because the former drugs produced more persistent falls of blood pressure.

Thirdly, vagotomy, which blocks the reflex actions of 5-HT and phenyl diguanide, did not abolish the antidiuretic action of 5-HT in 4 out of 6 cats. The effect of vagotomy on the antidiuretic action of 5-HT was difficult to assess, however, because after vagotomy the blood pressure usually rose, the rate of urine flow increased, and the response to 5-HT was usually pressor. The greater flow of urine due to increased blood pressure might explain why the antidiuretic effect of 5-HT was abolished by vagotomy in 2 of the cats and was considerably reduced by it in the others.

In another 3 experiments on cats, a large incision was made in the abdomen and part of the bowel pulled through the opening to permit direct observation of the right kidney and the ureter. Even when 5-HT was given in doses as large as 50 μg./kg., it did not cause paling of the kidney, nor did it throw the ureter into obvious spasm.

Experiments on Rabbits.—When the same kind of experiment was performed on 3 rabbits, the effect of 5-HT was much more pronounced. The paling of the kidney after large doses (20 to 50 μg./kg.) was striking and persistent. In 2 of the rabbits, the intravenous injection of 5-HT caused a rigor, more vigorous peristalsis, defaecation, and urination.

Experiments on Mice.—To see whether phenyl diguanide has an antidiuretic effect in mice, sub-

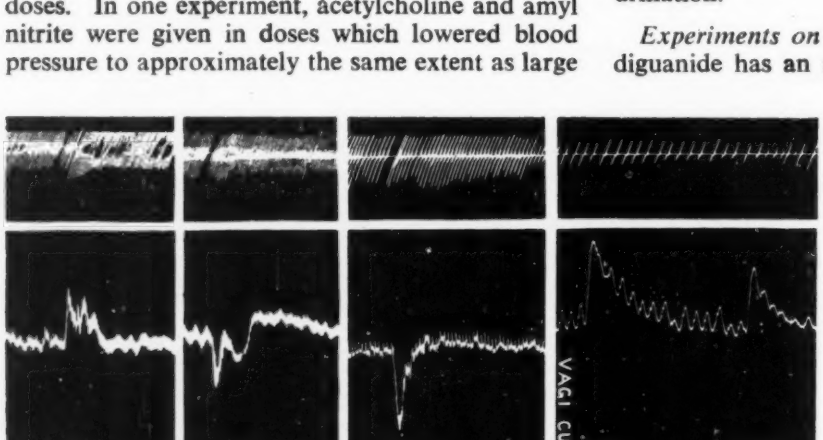

FIG. 2.—Cat. 3 kg. female. 210 mg. chloralose. Records from above downwards: respiratory movement, arterial blood pressure, urinary output, time (10 sec.). Note that 5-HT had a transient antidiuretic effect even when it produced a rise of blood pressure and after vagotomy. Phenyl diguanide (PhdiG) had a smaller antidiuretic effect despite the greater fall of blood pressure it produced.

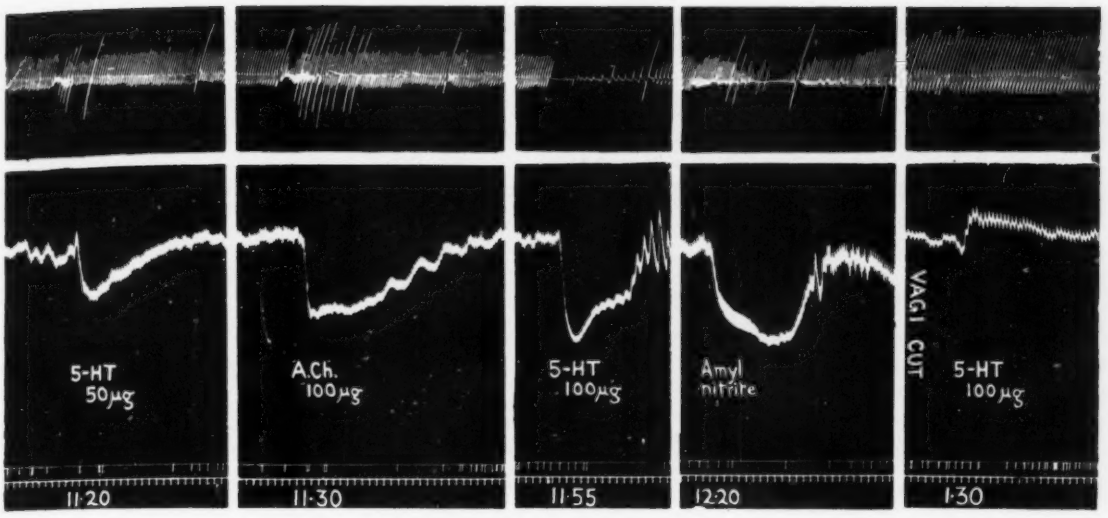

FIG. 3.—Cat. 3 kg. female. 200 mg. chloralose. Records from above downwards: respiratory movement, arterial blood pressure, urinary output, time (10 sec.). Note that 5-HT had a transient antidiuretic effect even when it produces a pressor response after vagotomy, and that acetylcholine (A.Ch.) and amyl nitrite (0.01 ml.) also have an antidiuretic effect when given in amounts producing substantial falls of blood pressure.

cutaneous doses of 0.05, 1, and 2.5 mg./kg. were given to hydrated mice. The amounts of urine excreted each ½ hr. up to 6 hr. after treatment were taken as the results for an analysis of variance. This analysis showed that neither the duplication nor the order of treatment had a significant effect. Nor was the average excretion rate, and hence the volume of fluid excreted over the total 6 hr. period, significantly altered by phenyl diguanide in the 3 concentrations tried. However, the two higher concentrations had a definite diuretic effect in that the peak diuresis at 1.5 hr. was significantly ($P<0.001$) higher than the peak diuresis for the controls, which also occurred 1½ hr. after the last dose of water.

DISCUSSION

In the cat small intravenous doses of 5-HT cause sharp falls of blood pressure in addition to decreasing urinary output for brief periods. Hypotension *per se* can have an antidiuretic effect. However, the antidiuretic effects which we observed cannot be attributed wholly or even mainly to the hypotensive action of 5-HT, because doses of phenyl diguanide which caused practically identical changes in blood pressure had little or even no antidiuretic effect. In the mouse, under conditions in which 5-HT shows strong antidiuretic activity (Waal and Veale, 1956), phenyl diguanide tended to increase rather than decrease urinary output.

The antidiuretic effects which 5-HT has been reported to produce in such animals as the rat and mouse are long-lasting (Erspamer, 1954 ; Waal and Veale, 1956), quite unlike those we have obtained in the cat. Now Abrahams and Pickford (1956b) have shown that small intravenous doses of 5-HT (5 to 20 μg.) exert such a powerful action on the ureteric muscle of the dog that the passage of fluid through the lumen of the ureter can be completely prevented for a minute or two and slowed for a further period of 1 to 2 min. Several findings support the idea that the antidiuretic effect of 5-HT in cats is due to ureteral spasm, such as the transience of the effect, the secondary diuretic effect of 5-HT in some cats (for urine held back by ureteral spasm would accumulate in the renal pelvis until the muscle relaxed, when a comparatively large volume at high pressure would be delivered in a short time), and the shorter duration of the antidiuretic effect with high rates of urine flow (for the greater the urine flow the sooner would a pressure be reached sufficient to force the ureter open). Nevertheless, when a length of ureter was observed directly, no noteworthy effect on tone could be detected after the injection of 5-HT.

In contrast to the cat kidney, the rabbit kidney became much paler when the animal was given a large intravenous dose of 5-HT. Moreover, the injection of 5-HT produced a number of other striking effects in the rabbit, due mainly it appeared to a sudden widespread increase in muscle tone.

Although the experiments on rabbits were not strictly comparable with those on cats in that a different anaesthetic was used and part of the alimentary tract was excised to give a good view of the kidney, it seems likely that the very different results obtained in the two series of experiments are due to species differences. Many of these have already been noted in studies with 5-HT ; some are mentioned earlier in this paper. Because of the likelihood of species differences, we hesitate to attribute the antidiuretic effects of 5-HT in the cat to either ureteral spasm or constriction of renal blood vessels, though we do not doubt the importance of these mechanisms in certain other species.

It is a pleasure to acknowledge our indebtedness to William R. Warner & Co. and to the Medical Research Council of New Zealand for financial assistance.

References

Abrahams, V. C., and Pickford, M. (1956a). *Brit. J. Pharmacol.*, **11**, 35.

—— —— (1956b). Ibid., **11**, 44.

Cerletti, A., Carpi, A., and Rothlin, E. (1955). *Helv. physiol. acta*, **13**, C8.

Corcoran, A. C., Masson, G. M. C., del Greco, F., and Page, I. H. (1954). *Arch. int. Pharmacodyn.*, **97**, 483.

Dawes, G. S., and Comroe, J. H. (1954). *Physiol. Rev.*, **34**, 167.

—— and Mott, J. C. (1950). *Brit. J. Pharmacol.*, **5**, 65.

Del Greco, F., Masson, G. M. C., and Corcoran, A. C. (1956). *Amer. J. Physiol.*, **187**, 509.

Erspamer, V. (1954). *Pharmacol. Rev.*, **6**, 425.

—— and Asero, B. (1952). *Nature, Lond.*, **169**, 800.

Gaddum, J. H. (1941). *J. Physiol.*, **99**, 247.

Kottegoda, S. R. S., and Mott, J. C. (1955). *Brit. J. Pharmacol.*, **10**, 66.

Page, I. H. (1952). *J. Pharmacol.*, **105**, 58.

—— (1953). *Circ. Research*, **1**, 354.

Sala, G., and Castegnaro, E. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 621.

Spinnazola, A. J., and Sherrod, T. R. (1955). *J. Pharmacol.*, **113**, 49.

Waal, H. J., and Veale, A. M. O. (1956). *Proc. Univ. Otago Med. Sch.*, **34**, 12.

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THE ETHYLENEIMINIUM ION AS THE ACTIVE SPECIES IN 2-HALOALKYLAMINE COMPOUNDS

BY

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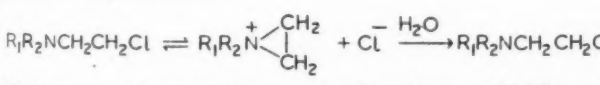
Certain 2-haloethylamine compounds which exert antagonism to adrenaline and noradrenaline of the type shown by dibenamine have been examined. The compounds were *N*-ethyl-*N*-chlorobenzyl-2-chloroethylamine hydrochlorides in which the Cl is in the *p*-, *o*-, and *m*- positions of the benzene ring, and the three related ethyleneiminium picrylsulphonates. In addition the Cl- and Br- compounds of the *N*-ethyl-*N*-9-fluorenyl-2-haloethylamine hydrohalide series were examined. For the haloethylamines, a curve was provided which showed the relation between probable production and decay of ethyleneiminium ion in neutralized solution with time. A correlation was established between the shape of this curve and the curve relating anti-adrenaline and anti-noradrenaline activity of samples of solutions of these compounds treated in an identical way. The relation to antagonism of histamine and 5-hydroxytryptamine was not so exact and varied with species.

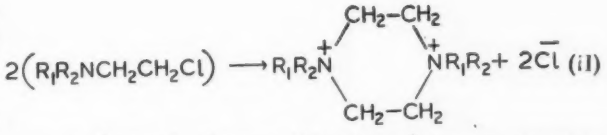
The ethyleneiminium ions of the three chlorobenzyl compounds exerted a typical long-lasting non-competitive antagonism to the pressor actions of adrenaline and noradrenaline. They also antagonized the effect of 5-hydroxytryptamine and histamine on blood pressure. The ED50 of the ion when administered as ethyleneiminium picrylsulphonate was always less than the ED50 of the same ion when administered in the form of 2-haloethylamine. There was, therefore, a variable loss of effective ion during transformation *in vivo*. The observations throw some light on the importance of the substituent groups on the nitrogen atom for effectiveness. It is concluded from these results and a discussion of other work that 2-haloethylamine compounds form an ethyleneiminium ion in neutral solution, which is the pharmacologically active species in effective compounds. It is a necessary but not a sufficient condition that a molecule should be an alkylating agent for this type of antagonism to be present, but the importance of the substituent groups on the N atom is equally great because not all ethyleneiminium ions are effective antagonists.

2-Chloroethylamine derivatives such as dibenamine have been shown to undergo the following reaction (I) when in solution in aqueous media at physiological *p*H, an unstable cyclized chemical species, the *N*-disubstituted ethyleneiminium cation being formed:



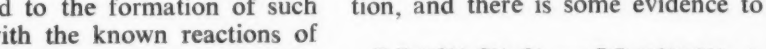
(I)

Nickerson, Nomaguchi, and Goodman (1946) attributed the specific pharmacological activity of this type of compound to the formation of such an ion, by analogy with the known reactions of the structurally related nitrogen mustards. Nickerson and Gump (1949) have shown that the hydrolysis products in such reactions as (I) are pharmacologically inactive. Intermediate products, such as the dimer which might be formed (II), were prepared and also found to be inactive.



(II)

It is theoretically possible for hydrogen chloride to be eliminated from the chloroethyl group with formation of a vinylamine (III), but it is improbable that such intermediates are formed from active 2-haloethylamines when in buffered solution, and there is some evidence to the contrary.



(III)

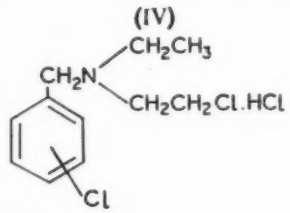
Such a compound would probably not react with sodium thiosulphate. Chapman, James, Graham, and Lewis (1952) reported the isolation and chemical characterization of the ethyleneiminium

ion derived from *N*-methyl-*N*-2-naphthylmethyl-2-bromoethylamine as a picrylsulphonate, but as this compound was insoluble in aqueous media it was unsuited to biological investigation. Accordingly lines of investigation were followed which did not involve the isolation of active cations from the parent compounds.

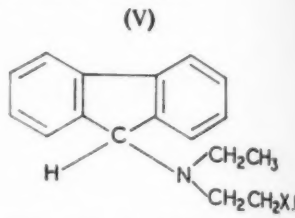
It was known that primary alkyl fluorides undergo substitution with much less ease than do other alkyl halides. A series of *N* - alkyl - *N* - 1 - naphthylmethyl - 2 - halo - ethylamines was prepared in which the halogen was Cl, Br, I or F (Chapman, James, Graham, and Lewis, 1951 ; Chapman and James, 1953) and it was shown that the fluorine compounds were pharmacologically and chemically inert, whereas the other halogen compounds were active. Mobility of the halogen is thus necessary for adrenergic blocking activity (Graham and Lewis, 1952, 1953). Chapman *et al.* (1952) and Chapman and James (1954) studied the kinetics of decomposition of some of these compounds in initially neutral solution and published curves relating the amount of thiosulphate consumed with the passage of time. Sodium thiosulphate reacts rapidly and stoichometrically with the ethyleneiminium ion; its consumption has been generally accepted as a measure of the amount of such ion in a mixture of products. The time relations of the release of halogen in the reaction mixture, the amounts of thiosulphate consumed, and the release of hydrogen ion were determined and plotted in graphic form. The pharmacologically inert fluorine-containing compounds were chemically inert and no halogen was released or thiosulphate consumed. In this respect, therefore, the conditions which determine cation formation also determine pharmacological activity. The presence and amount of anti-adrenaline and antihistamine activity was determined in solutions of *N*-ethyl-*N*-1 - naphthylmethyl - 2 - haloethylamine (halogen=Cl, Br or I), prepared and handled in a manner identical to that used for the solutions in which chemical reactivity had been studied. It was thus possible to relate the formation and loss of a thiosulphate-consuming species (ethyleneiminium ion) in these solutions with specific pharmacological activity. It was clear that antagonism to adrenaline and histamine and consumption of thiosulphate ran a parallel course in these solutions as they were allowed to age (Graham and Lewis, 1954).

To exclude the possibility of this relationship being peculiar to these particular haloethylamine compounds it was decided to extend the examination to include further compounds, the *N*-ethyl-*N*-chlorobenzyl-2-chloroethylamines (IV) with Cl in the *ortho*- (JFA14), *meta*- (JFA11), or *para*- (JFA8) position of the benzyl residue, and *N*-ethyl-*N*-9-fluorenyl-2-haloethylamines (V), in which the halogen was Cl (AT_1) or Br (AT_2).

(IV)

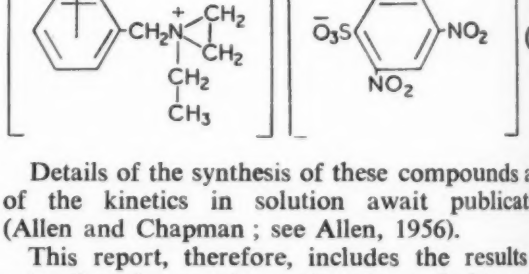


(V)



Nickerson and Gump (1949) refer briefly to two of these compounds, JFA11 and JFA8, and note that the former is more active. Kerwin, Herdegen, Heisler, and Ullyott (1950) describe the synthesis of compound AT1, and Kerwin, Ullyott, Fellows, and Macko (1949) state that it inhibits the pressor response to adrenaline in cats and is more active than dibenamine.

As this line of enquiry came to an end the ethyleneiminium ions derived from the three *N*-ethyl-*N*-chlorobenzyl-2-chloroethylamines became available in stable soluble form as picrylsulphonates (VI). They were given the code numbers P_1 (*p*-chloro-), P_2 (*m*-chloro-), and P_3 (*o*-chloro-) cations.



(VI)

Details of the synthesis of these compounds and of the kinetics in solution await publication (Allen and Chapman ; see Allen, 1956).

This report, therefore, includes the results of extending the enquiry, using the technique described by Graham and Lewis (1954), and the examination of the specific activity of the ethyleneiminium ions derived from selected active halogenoethylamine compounds. A preliminary communication has been made by Graham (1957).

Methods

Chemical.—The chemical investigation was carried out by Allen and Chapman. Briefly, it consisted of the preparation of 200 ml. of 0.02 M solution of the compound in acetone-water (2:1). The finely powdered salt (4 mM.) was dissolved in a mixture of water (50 ml.) and acetone (132 ml.) at 30°, 10 ml. of 0.4 M solution of NaOH (4 mM. NaOH) added.

the solution made up to 200 ml. with water, shaken and kept at 30°. Aliquots were withdrawn at intervals and washed twice with ether to remove unchanged parent compound. Free halide, H ion, and ethyleneiminium ion were then determined. The % of the theoretically possible reaction (as g.-ion/mole reactant, maximum possible ion 0.004 mole) was plotted against time.

In addition the rate of loss of ion was determined when the aqueous phase after ethereal extraction of the mixture was kept on ice for periods of 10 to 72 min. This observation was made on mixtures of various ages up to 4 hr. and the loss was found to be negligible over a period of 60 min.

Pharmacological.—The solutions used were prepared in a manner identical to the above but adapted to give 10 ml. volume. 0.2 mM. of the compound was dissolved in 3 ml. water plus 6 ml. acetone, and 1 ml. N/5 solution of NaOH added, made up to 10 ml. volume and kept at 30°. At the selected time the mixture was extracted twice with 2 vol. of ether and the watery phase stored on ice while the ED50 was determined. Ten dogs were used for the solutions of that age where peak activity was found by preliminary trial (the reference point plotted as 100% activity) and 5 for the solutions of other ages examined. Five compounds were examined in this way. The dogs were atropinized, anaesthetized with 30 mg./kg. pentobarbitone sodium intravenously, and the blood pressure recorded. Repeated injections of (−)-adrenaline 4 μg./kg., equimolar amounts of (−)-noradrenaline and of histamine, and in some dogs 5 times this amount of 5-hydroxytryptamine (5-HT) were made into the jugular vein. At suitable intervals a dose of the treated solution of haloethylamine was injected. This was allowed to act for 15 min. and the standard injections repeated in random order. It had previously been shown that the parent compounds in solution exerted the maximum effect of any given dose in 15 min. or less and maintained this effect for at least 4 to 6 hr. The action of the individual doses was assumed to be cumulative. The amount given in mg./kg. weight of dog was recorded in terms of amount of parent compound present initially in the solution (0.02 M). The experiments were allowed to proceed until non-competitive reversal of the pressor responses was obtained, and, when possible, until partial recovery was noted. Less than 1% loss of activity occurs in an ice-cold solution of "ion" kept for 1 hr. Fresh solutions were prepared for subsequent stages of the experiment. The pharmacological activity of solutions of different ages was expressed as % of the mean activity measured at the age when peak activity was found to be present.

The ethyleneiminium picrylsulphonates were dissolved in a minimal volume of acetone (0.1 ml. for rats) and diluted as needed with saline. The ED50 for each of the 5 compounds and the 3 ethyleneiminium picrylsulphonates was determined against the 4 agonists in groups of 5 dogs and in 30 male atropinized spinal hooded rats pretreated with 5 mg./kg. of hexamethonium intravenously. Antagonism to adrenaline (1 μg./kg.) and to noradrenaline (0.5 μg./kg.) was measured together; antagonism to 5-HT (10 μg./kg.) was determined on different rats. Histamine was excluded from this series. Five rats were used for each determination and the parent compounds were similarly investigated for comparison.

The effect of Na picrylsulphonate by itself and on the responses to the four agonists was determined over a wide range of doses, in 3 dogs and 10 spinal rats, as a necessary preliminary to this part of the work.

Results

The Relationship Between Ion Production and Pharmacological Activity

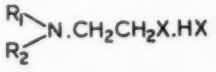
Some properties of the compounds examined are shown in Table I, which was compiled from information supplied by Allen and Chapman.

Compounds JFA8, JFA11, and JFA14 were freely soluble in water. Compounds AT1 and

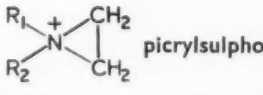
TABLE I

THE STRUCTURE, CODE NUMBERS AND MOLECULAR WEIGHTS OF THE COMPOUNDS AND IONS REFERRED TO IN THE TEXT

Solubility of AT_1 and AT_2 in water is poor, but they are soluble in acetone and this may be diluted 10 times with water before a precipitate appears. The ions P_1 to P_3 are similarly insoluble in water but soluble in acetone and may be diluted 19 times with water before precipitating. The last column shows the time of peak concentration of ion found by titration with thiosulphate in initially neutralized solutions of the haloethylamines and the maximum conversion to ion as a % of the theoretically possible figure.



R_1	R_2	X	Code No.	M.W.	Vol. of Water Dissolving 1 g. (ml.)	Max. Conversion to Ion as % of Theoretical Maximum
Et	*p*-Chlorobenzyl	Cl	JFA8	268·6	3·42	36·0 at 30 min.
,,	*m*-Chlorobenzyl	Cl	JFA11	268·6	6·34	26·1 at 30 min.
,,	*o*-Chlorobenzyl	Cl	JFA14	268·6	1·50	22·0 at 45 min.
,,	9-Fluorenyl	Cl	AT1	308·2	Sol. very poor	7·0 at 15 min.
,,	9-Fluorenyl	Br	AT2	397·2		40·0 at 0·5 min.



picrylsulphonate

R_1	R_2		Code No.	M.W.	Vol. Acetone Dissolving 1 g. (ml.)	Max. Conversion to Ion as % of Theoretical Maximum at Zero Time
Et	*p*-Chlorobenzyl		P1	488·9	77	100
,,	*m*-Chlorobenzyl		P2	488·9	50	100
,,	*o*-Chlorobenzyl		P3	488·9	45	100

AT2 were relatively insoluble, but solutions in small volumes of acetone were easily diluted with water. The picrylsulphonates (P_1 to P_3) were also rather insoluble in water but readily soluble in acetone and did not easily come out of solution on dilution with water or saline. The amount of ion formation actually found on titration was recorded as a % of the theoretical maximum. The time taken to reach this value was noted for each compound.

Table II shows the values of the ED50 in mg./kg. of the five compounds, prepared in neutralized solution as described, kept at 30° for the time intervals shown, extracted with ether, kept on ice, and determined. The height of the pressor responses was measured in mm. and % inhibition of the mean initial response plotted against the log-dose of antagonist compound. The values of the ED50 were derived from these plots and were used to determine the points on the curves of Figs. 1 to 5.

Table II shows that antagonism to adrenaline was more marked than that against noradrenaline. Antagonism to histamine and to 5-HT was more variable, but usually 5-HT was less easily antagonized than noradrenaline and more easily antagonized than histamine. In some dogs small doses of the 2-haloalkylamine compounds potentiated the pressor response to injected adrenaline. Potentiation of histamine and 5-HT was more marked and more frequent, and it happened after much higher doses of compound. On occasions this led to a persistent increase rather than a decrease in the response to histamine and 5-HT: the phenomenon was never encountered with adrenaline or noradrenaline. In some dogs, particularly with compound AT2, no accurate determination of the ED50 against histamine and 5-HT could be made because potency was feeble. The times at which maximum amounts of ethyleneiminium ion were found in the kinetic studies were the times at which greatest biological activity was usually detected. Activity varied along the time scale in a manner similar to the variation in concentration of ion. This relation was more precise for antagonism to adrenaline and noradrenaline than to histamine and 5-HT. In the instance of 5-HT, lack of correlation may be due in part to the variation in dogs of the responses to repeated injections of standard amounts of amine.

The relation between the amount of titratable ethyleneiminium ion in solutions of these compounds and pharmacological activity is clearer if we plot ion production and activity at the various points in the time scale examined as % of the peak values obtained. These plots for the five compounds and the various amines are shown in Figs. 1 to 5.

Chloro-benzyl Derivatives.—The relation between the curves of production and decay of ethyleneiminium ion and potency as an antagonist of the pressor activity of adrenaline and nor-

TABLE II

MEDIAN EFFECTIVE DOSES OF NEUTRALIZED SOLUTIONS OF 2-HALOETHYLAMINES OF DIFFERENT AGE

The Table shows the relation between the time in min. for which a neutralized solution of 2-haloethylamine is kept at 30° and the ED50 in mg./kg. body weight in dogs anaesthetized with pentobarbitone sodium. The median effective dose was determined against (−)-adrenaline 4 μg./kg. (A), an equimolar amount of (−)-noradrenaline (N), or histamine (H), and five times as much 5-hydroxytryptamine (HT). The sign + indicates that the effect of the agonist was potentiated. An asterisk indicates the point in the time scale at which peak production of ion occurred as determined in kinetic studies, except in the case of compound AT_2, in which reactivity was so great initially that an arbitrary point at 10 min. was chosen.

N-Ethyl-N-chlorobenzyl-2-chloroethylamine Hydrochlorides

Time (min.)	JFA8			Time (min.)	JFA11			Time (min.)	JFA14			
	A	N	H		A	N	H		A	N	H	HT
1	1·90	4·00	5·86	1	0·85	0·93	2·95	1	1·97	4·17	14·5	1·43
10	0·71	1·26	2·80	10	0·13	0·21	0·39	10	0·28	0·50	+	+
*30	0·61	1·01	1·21	*30	0·11	0·17	0·50	*50	0·26	0·38	1·31	0·50
120	0·85	1·53	2·30	120	0·13	0·19	6·30	120	0·37	0·68	1·59	1·51
240	0·90	2·00	4·65	240	0·20	0·28	1·04	240	0·70	1·21	2·40	0·78

N-Ethyl-N-9-fluorenyl-2-haloethylamine Hydrohalides

Time (min.)	AT_1				Time (min.)	AT_2			
	A	N	H	HT		A	N	H	HT
3	0·21	0·37	2·17	0·96	0·5	0·09	0·22	1·65	5·28
*15	0·073	0·16	2·0	0·25	*10	0·15	0·40	1·27	1·13
30	0·077	0·18	>3·0	5·4	30	0·27	0·66	+	>10
60	0·10	0·25	6·0	2·27	60	0·75	1·30	14·1	>10
120	0·18	0·44	16·7	2·18	120	3·0	4·0	9·8	>10
240	0·24	0·79	8·3	2·50					

adrenaline was particularly close with the *m*- and *p*-chlorobenzyl compounds (Figs. 1 and 2; compounds JFA8 and JFA11) and rather less so with the *o*-chloro compound (Fig. 3; compound JFA14). The relation became less close and the

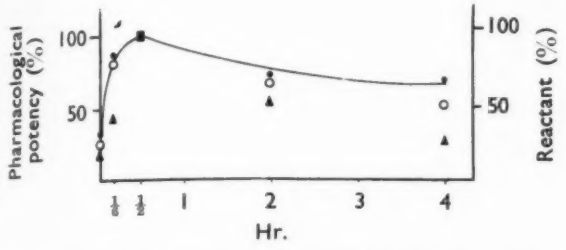


FIG. 1.—The relation between chemical reactivity and pharmacological activity in *N*-ethyl-*N*-*p*-chlorobenzyl-2-chloroethylamine HCl (JFA8). The line represents the variation with time of the production and decay of ethyleneiminium ion expressed as % of the maximum found at the point of peak activity (■, right-hand ordinate). The pharmacological potencies are expressed as % of the peak activities, measured as ED50 on the blood pressure of dogs anaesthetized with pentobarbitone sodium 30 mg./kg. (left-hand ordinate). ● represents the antagonism to (−)-adrenaline, 4 μg./kg.; ○ represents the antagonism to equimolar amounts of (−)-noradrenaline; ▲ gives the antagonism to equimolar histamine.

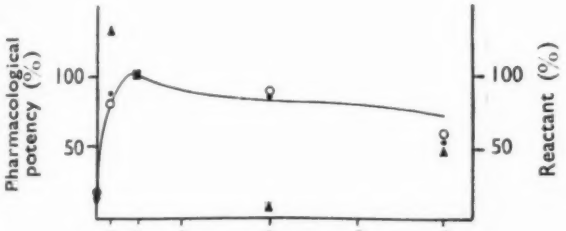


FIG. 2.—The relation between production of ethyleneiminium ion and antagonism to adrenaline (●), noradrenaline (○), and histamine (▲) in *N*-ethyl-*N*-*m*-chlorobenzyl-2-chloroethylamine HCl (JFA11). See Fig. 1 for explanation. There is no close correlation with antagonism to histamine.

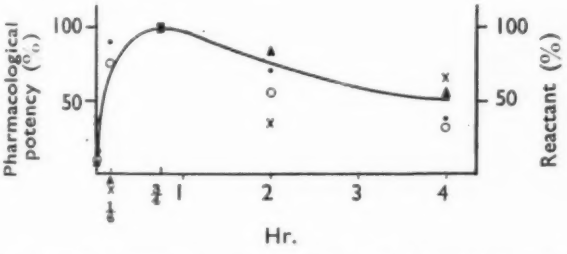


FIG. 3.—The relation between production of ethyleneiminium ion and antagonism to adrenaline (●), noradrenaline (○), histamine (▲), and 5 times equimolar 5-hydroxytryptamine by *N*-ethyl-*N*-*o*-chlorobenzyl-2-chloroethylamine HCl (JFA14). See Fig. 1 for explanation. The sign X represents antagonism to 5-HT. The responses to histamine and 5-HT were potentiated by all doses of the ionized solution aged 10 min. in 3 of the 5 dogs used, and are shown below the baseline.

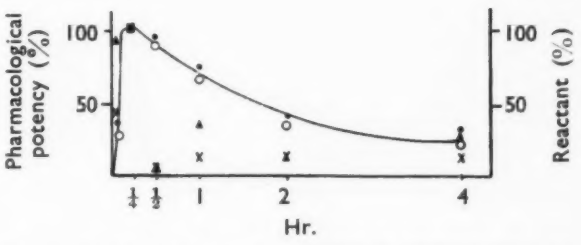


FIG. 4.—The relation between production of ethyleneiminium ion and antagonism to adrenaline (●), noradrenaline (○), histamine (▲) and 5-HT (X), by *N*-ethyl-*N*-9-fluorenyl-2-chloroethylamine HCl (AT1). See Fig. 1 for explanation. There is no correlation with antagonism to histamine or 5-HT.

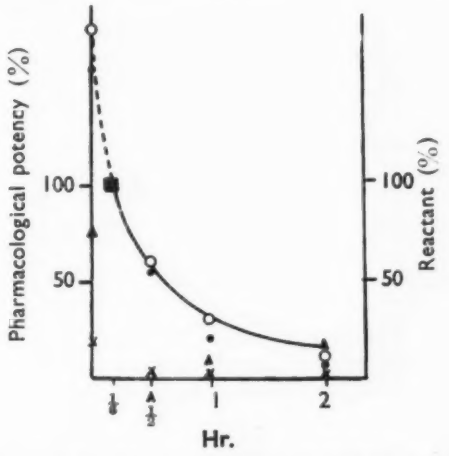


FIG. 5.—The relation between production of ethyleneiminium ion and antagonism to adrenaline (●), noradrenaline (○), histamine (▲), and 5-HT (X), by *N*-ethyl-*N*-9-fluorenyl-2-bromoethylamine HBr (AT2). See Fig. 1 for explanation. Due to the speed of reactivity, the levels measured after 10 min. reaction were expressed as 100%; the points on the curve of reactivity prior to that time were established less critically and are expressed by an interrupted line. They are not extrapolations but approximate measures. The correlation with antihistamine activity is poor; there is no correlation with the low level of activity against 5-HT shown by this compound.

scatter of individual values increased in solutions 4 hr. old. A causal relationship between the amount of ion in solution and antagonism to the hypotensive action of histamine was not established for the *m*-chlorobenzyl compound (Fig. 2; compound JFA11), but it may exist with the other two compounds. Likewise, the relation of ion production with antagonism to the pressor response to 5-HT (Fig. 3; compound JFA14) was not close although the general trend was for the two properties to move together.

9-Fluorenyl Derivatives.—The correspondence between ion production and decay with time and antagonism to adrenaline and noradrenaline was close for the chloro- and bromo-9-fluorenyl compounds AT1 and AT2 (Figs. 4 and 5), but again

was much less close for antagonism to histamine and 5-HT. There was no reduction in antagonism to adrenaline and noradrenaline relative to ion content of the solutions with ageing at 4 hr. as was seen with the chlorobenzyl compounds.

Activity of N-*Ethyl*-N-*Chlorobenzyl Ethyleneiminium Picrylsulphonates*

Sodium picrylsulphonate in doses up to 20 mg./kg. injected intravenously did not alter the basal blood pressure of the anaesthetized dog and had no effect on the response to repeated injections of adrenaline, noradrenaline, histamine, and 5-HT. In the spinal rat, the result was not quite so clear. It was impracticable to examine quantitatively the responses to adrenaline, noradrenaline and 5-HT on one such preparation because the administration of 5-HT modified the responses to the other two amines. Sodium picrylsulphonate in doses up to 32 mg./kg. did not alter the blood pressure but had a variable effect on the pressor responses to the amines, particularly 5-HT. In most rats, the responses to repeated injections of adrenaline and noradrenaline, or of 5-HT, were not altered by less than 1 mg./kg. of picrylsulphonate, which corresponded to a greater amount than was present in the ED50 of the ions P1 to P3. With amounts greater than 1 mg./kg. the responses were nearly always potentiated, which meant that the slopes of the dose response lines from which the ED50 values were derived tended to be less steep at higher dose levels of the antagonists and the ions thus appear to be less effective than they were. This source of error was never more than 15% of the initial response, was unpredictable, and its net result was to diminish rather than to exaggerate the difference in activity of a compound administered as salt of ion or as salt of parent compound; it was therefore ignored.

It was not possible to obtain solutions of the ions as picrylsulphonates which consumed more than 94% of the theoretically possible amount of thiosulphate, despite apparent analytical purity and constant melting point (Allen and Chapman). The solutions therefore contained only 94% of active ion credited to them. This factor also tended to make the ions appear to be less effective.

Fig. 6 depicts the effect of injections of a solution of the picrylsulphonate of one of these ions (P3) on the blood pressure of a dog anaesthetized with pentobarbitone sodium. Doses which reversed the pressor response to adrenaline had no effect on the resting blood pressure; larger doses, which abolished the pressor response to 5-HT and greatly diminished or abolished the response to noradrenaline, lowered the resting blood pressure only slightly. The effect on the response to histamine was slight and not related to the dose of compound administered. The general pattern of responses was very similar to that produced by the parent haloethylamine.

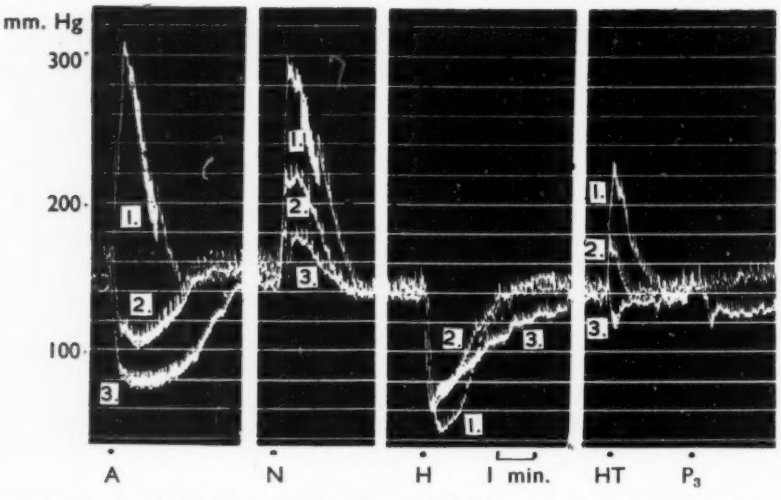


FIG. 6.—Dog, male, 11.3 kg., pentobarbitone sodium 30 mg./kg. i.v., atropine 3 mg. s.c. Record of the carotid blood pressure. The panels record the effect of (−)-adrenaline, 4 μg./kg. (A1), equimolar (−)-noradrenaline (N1), equimolar histamine (H1), and 5 times equimolar 5-HT (HT1). Two injections of 0.30 mg./kg. of *N*-ethyl-*N*-*o*-chlorobenzyl ethyleneiminium picrylsulphonate (P3), given at an interval of 30 min. (P_3 2 and 3) were allowed to act for 5 min. and the standard injections repeated. The basal pressure was slightly reduced (P_3 3), the response to adrenaline reversed (A2 and 3), that to noradrenaline reduced (N2 and 3), that to histamine slightly reduced (H2 and 3), that to 5-HT reduced or reversed (HT2 and 3).

The actions and potencies of the ethyleneiminium ions derived from the three chlorobenzyl compounds and of the parent compounds are recorded in Table III.

Table III reveals several points of interest. The three ions antagonized the changes in the blood pressure of the anaesthetized dog and spinal rat brought about by injection of adrenaline, noradrenaline, histamine, and 5-HT. There were species differences in these activities. The *p*-chlorobenzyl ion, P1, was less active than the *m*- and *o*-chlorobenzyl ions P2 and P3, and its parent compound JFA8 less active than the *m*- and *o*-chlorobenzyl compounds JFA11 and JFA14. There was not much difference in the potencies of P2 and P3 ions, but considerable

TABLE III

THE VALUES OF THE ED50 FOR THE ETHYLENEIMINIUM IONS AND FOR THE PARENT COMPOUNDS

The values of the ED50 mg./kg. of three ethyleneiminium ions and of parent compounds which antagonize the response of the blood pressure to adrenaline (A), noradrenaline (N), histamine (H), and 5-hydroxytryptamine (HT) in the spinal rat and the anaesthetized dog. The estimates are corrected in terms of % of ion available. (c) indicates that the antagonism was weak and the value of the ED50 was only approximate. The sign — indicates that the substance was not tested. The values for the ED50 of the parent compounds are expressed in terms of the maximum theoretical amount of ion available if the compound were fully ionized, and also in terms of the maximum actually found at the time of peak concentration of ion as determined by consumption of thiosulphate. The ratios of the values for the ED50 of the ions which were given as picrylsulphonates and of the ions which were available from the parent compounds are also shown.

	(1) Ion as Picrylsulphonate (P)							Available Ion Pure (1a)						
	Rat			Dog				Rat			Dog			
	A	N	HT	A	N	H	HT	A	N	HT	A	N	H	HT
P_1 ..	0·60	0·84	1·10	1·30	2·40	2·90	1·00	0·240	0·336	0·440	0·520	0·960	1·16	0·400
P_2 ..	0·42	0·57	0·98	0·22	0·37	5	0·19	0·168	0·228	0·392	0·088	0·148	2	0·076
P_3 ..	0·32	0·48	0·78	0·20	0·42	5	0·60	0·128	0·192	0·312	0 080	0·168	2	0·240
	(2) Parent Compound as Hydrochloride							Available Ion (Max. Theoretical) (2a)						
JFA8 ..	9·80	10·60	16·2	6·1	7·9	c 9·0	—	7·15	7·73	11·8	4·45	5·76	c 6·5	—
JFA11 ..	1·31	1·35	6·3	4·0	6·2	c 12	—	0·95	0·98	4·6	2·92	4·52	c 8·7	—
JFA14 ..	6·43	5·93	10·2	1·6	2·8	c 16	—	4·69	4·32	7·4	1·16	2·04	c 11·6	—
	Approx. Ratio ED50. $\frac{\text{Ion Found from Parent}}{\text{Pure Ion (P)}} = \frac{2b}{1a}$							Available Ion (Max. Found) (2b)						
JFA8/P1	10	9	10	3	2	2		2·570	2·780	4·25	1·60	2·07	2·34	—
JFA11/P2	1·5	1·1	3	8	7	1·13		0·247	0·255	1·19	0·76	1·17	2·26	—
JFA14/P3	8	5	5	3	2·6	1·25		1·030	0·950	1·62	0·25	0·44	2·50	—

difference in the potencies of their parent compounds JFA11 and JFA14, particularly in the rat. The number of animals used in each test (5 rats and 5 dogs) seemed adequate, and sufficient time was allowed for the parent compound to exert maximum effect before testing. The antagonism of the ions and their parent compounds to adrenaline was more marked than their antagonism to noradrenaline. The pattern of antagonism to histamine and 5-HT was less consistent. In the rat, 5-HT was antagonized less than noradrenaline by the ions and their parent compounds; in the dog 5-HT was antagonized more than histamine by the ions. The parent compounds were not active antagonists of histamine; 5-HT was not tested. Potentiation effects were not so marked as with the treated solutions of parent compounds (see Table II) and did not prevent the development of inhibition of the responses to histamine and 5-HT. Such effects were, however, present in some animals with some doses of either ion or parent compound in relation to any one of the agonists. The ED50 of the ion, expressed either as weight/kg. of its picrylsulphonate or as available ion, was always less than the ED50 of the parent compound, expressed as weight of halogen hydracid salt, or as available ion in terms of the theoretically possible maximum, or in terms of % found *in vitro* by consumption of thiosulphate. For both species of test animal and all 4 agonists the ratio of these active amounts (ED50) was in all cases greater than unity, but there was a wide scatter in the indices.

DISCUSSION

The evidence provided in Table II and the graphic presentation in Figs. 1 to 5 make it clear that a quantitative relationship exists between the anti-adrenaline and anti-noradrenaline activity of these 2-haloethylamine compounds and the amount of ethyleneiminium ion present in the solution. With antihistamine activity, the relationship is reasonably good for two of the chlorobenzyl molecules and poor for the other; there is no such relationship for the two 9-fluorenyl compounds. With anti-5-HT activity, the relationship is discernible with the only chlorobenzyl compound examined but not with the 9-fluorenyl compounds.

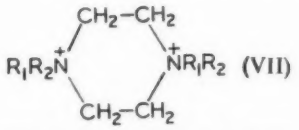
We may therefore conclude with some degree of confidence that over a wide range of structure (three *N*-chloro-benzyl haloethylamines and two *N*-9-fluorenyl-haloethylamines reported above, and three *N*-naphthylmethyl haloethylamines examined similarly by Graham and Lewis, 1954) there is a direct proportionality between the antagonism to adrenaline and to noradrenaline and the concentration of thiosulphate-consuming species and a limited relationship to antihistamine and anti-5-HT activity. The latter relationship varies with the structure of the compound examined. In addition

there is the evidence provided by Chapman and James (1953) and Graham and Lewis (1953) in kinetic and pharmacological studies that, if the halogen in the alkyl chain is fluorine, compounds are non-reactive chemically and inactive pharmacologically, whereas the substitution of any other halogen for fluorine restores both properties to a similar degree in these molecules.

If the active haloethylamines are dissolved in an aqueous medium, the base may be liberated from the salt and left for an appropriate time. The unchanged parent compound may then be extracted from the mixture by appropriate treatment with ether, and the residue still contains a thiosulphate-consuming species. The formation of acid can be followed during the whole of the reaction, particularly in its early stages. With a compound which liberates the whole of its halogen immediately and gives a corresponding amount of ethyleneiminium ion there is scarcely any initial acidity. This was shown by Chapman *et al.* (1952) and Chapman and James (1954). The intermediate formed is therefore likely to be an ethyleneiminium ion and not a vinylamine.

The drift away from a close correlation between anti-adrenaline and anti-noradrenaline activity and the curve of decay of ion in initially neutral solutions of compounds JFA8 and JFA14, and to a lesser extent JFA11, has no theoretical explanation but may be due to the fact that neutralized solutions of the ages involved (4 hr.) were relatively inactive and usually required a greater number of injections in order to establish a satisfactory dose/response relationship. The experimental design of the work was such that the three solutions of age 4 hr. were investigated before it was realized that decay of the ion in neutral aqueous solution would take place to an appreciable extent at room temperature if there was delay beyond 60 min. in establishing a result. The potencies at these points could probably be raised by 10% with justification, but the correction has not been made because there is no precise record of the time for which the samples were kept before injection. All subsequent work was done with the aqueous solutions kept on ice. No such discrepancy was observed by Graham and Lewis (1954) with the naphthylmethyl compounds and the phenomenon is probably devoid of true significance.

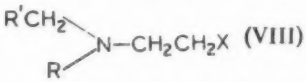
The picrylsulphonates of ethyleneiminium ions were prepared by precipitation at 0° by acid sodium picrylsulphonate of a neutralized solution in acetone water of the 2-haloethylamine salt concerned (Chapman and James, 1954; Allen, 1956), and ultimately analysed. It has been suggested that the dimer or piperazinium ion (VII),



(VII)

would give rise to the same ultimate analysis as the ethyleneiminium ion and might be the active species. There are two points of evidence against this. (1) The supposed ethyleneiminium ions (compounds P1 to P3) consume thiosulphate to an extent of 94% of the theoretical maximum. Simple piperazinium compounds do not consume thiosulphate; piperazinium ions from active 2-haloalkylamines have not been tested. (2) The piperazinium salts from certain active 2-haloalkylamines (including compound *N*-ethyl-*N*-naphthylmethyl-2-chloroethylamine HCl [J10 in the list of compounds examined by Graham and Lewis, 1953, 1954] have been made, tested, and reported inactive by Nickerson and Gump (1949). The picrylsulphonate compounds P1 to P3 are very active and the compound J10 is active.

Chapman *et al.* (1952) have shown that for the series of naphthylmethyl-2-haloethylamine derivatives (VIII) where R=phenyl, R′=1- or 2-naphthylmethyl and X=Cl, Br or I, there



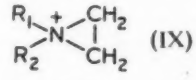
(VIII)

is no consumption of thiosulphate, very little liberation of halide ion, and no formation of ethyleneiminium ion. Graham and Lewis (1953) found that these compounds were inactive. Ing (1956) has pointed out that the phenyl group attached directly to the *N* atom reduces the basicity of the amine sufficiently to prevent intramolecular alkylation (see Chapman and James, 1953).

Peart (1956) has objected that the evidence of Harvey and Nickerson (1953) as to the relation of chemical transformation of dibenamine and dibenzyline to biological activity in these compounds is only indirect. A biologically inert β-haloalkylamine (*N*:*N* - dicyclohexyl - β - chloroethylamine) gave the same indirect chemical reactions (evolution of Cl^- and H^+ and consumption of thiosulphate) which are supposed to indicate ethyleneiminium ion formation. He continues: "The fact that thiosulphate could prevent the block if it was administered before, or simultaneously with, the β-haloalkylamine was used as evidence that such an intermediate was formed, since it was known

that thiosulphate reacts with similar derivatives of nitrogen mustards. However, it will also probably react directly with unchanged β-haloalkylamine so that overall the evidence in favour of an active intermediate is inconclusive."

As to the first point, it has never been suggested that all structures of the ethyleneiminium type are active anti-adrenaline compounds, though all active 2-haloethylamines are alkylating agents. Much depends on the conditions to be fulfilled by the substituent groups R_1 and R_2 in the ion (IX).


(IX)

All active anti-adrenaline compounds of the dibenamine type are necessarily alkylating agents, but it does not follow that all alkylating agents are anti-adrenaline compounds. The importance of the substituent on the nitrogen atom is illustrated in the results of the present investigation. The order of potency of the *N*-ethyl-*N*-chlorobenzyl-2-chloroethylamine HCl compounds is found to be JFA11>JFA14>JFA8 in the spinal rat, which technically is the better preparation in which to measure antagonism to pressor substances. The order of availability of ion *in vitro* from these compounds is JFA8>JFA11>JFA14. The order of potency in the same animal preparation of the ions derived from these compounds is ion of JFA14>ion of JFA11>ion of JFA8. In addition, in all cases the injection of the ion as a picrylsulphonate into the blood stream proves to be more potent than the injection of the same available amount of ion when it has to be derived by cyclization from its parent haloethylamine and the indices of relative activity vary with species and agonist (Table III). It follows that the factors controlling solubility and reactivity of the parent compound *in vivo* may be as important to its effectiveness after administration as the potency of the pure ion. The haloethylamine which reacts to the greatest extent in neutral solution (JFA8) is the least potent because the effects of the group R^2 are such as to give least potency as an antagonist; the haloethylamine with the most potent ion structurally (JFA14) cyclizes to the least extent and is therefore of only moderate potency. The most potent is such that it cyclizes well and produces an effective ion. The rate of ionization in buffered protein-containing medium is obviously of great importance.

As to the second point discussed by Peart (1956), it is likely that thiosulphate will react with parent halogenoalkylamine, but this is difficult to prove because it is always possible for the parent substance to form the ethyleneiminium ion and react by that route. If the halogen is inert, as in fluorine compounds, no thiosulphate is consumed, and there is no biological activity.

The evidence provided in this paper in conjunction with the investigations of Chapman and his colleagues on kinetics show that antagonism to adrenaline and noradrenaline is dependent on production of ethyleneiminium ion at an effective rate, in adequate amounts, and of a proper structure. It does not throw light on the relationship between structure, ion formation, lipoid solubility and duration of effectiveness discussed by Nickerson (1949), Hunt (1949), and Axelrod, Aronow, and Brodie (1952), but makes it clear that these criteria must be observed before the other matter can be properly discussed. Dibenamine is not the best substance for such studies.

I am glad to acknowledge the technical assistance of Mr. C. F. Lee and Mr. D. Salway. As usual, Dr. N. B. Chapman and colleagues have collaborated closely.

References

Allen, J. F. (1956). Ph.D. Thesis, Univ. of Southampton.

Axelrod, J., Aronow, L., and Brodie, B. B. (1952). *J. Pharmacol.*, **106**, 166.

Chapman, N. B., and James, J. W. (1953). *J. chem. Soc.*, 1865.

—— —— (1954). Ibid., 2103.

—— —— Graham, J. D. P., and Lewis, G. P. (1951). *Chem. & Ind.*, 633.

—— —— —— —— (1952). Ibid., 805.

Graham, J. D. P., and Lewis, G. P. (1952). *J. Physiol.*, **116**, 37*P*.

—— —— (1953). *Brit. J. Pharmacol.*, **8**, 54.

—— —— (1954). Ibid., **9**, 68.

—— (1957). *J. Physiol.*, **135**, 62P.

Harvey, S. C., and Nickerson, M. (1953). *J. Pharmacol.*, **109**, 328.

Hunt, C. C. (1949). Ibid., **95**, 177.

Ing, H. R. (1956). *Hypotensive Drugs*, ed. M. Harington, p. 11. London: Pergamon Press.

Kerwin, J., Ullyott, G. E., Fellows, E. J., and Macko, E. (1949). *Fed. Proc.*, **8**, 308.

—— Herdegen, T. F., Heisler, R. Y., and Ullyott, G. E. (1950). *J. Amer. chem. Soc.*, **72**, 940.

Nickerson, M. (1949). *Pharmacol. Rev.*, **1**, 27.

—— Nomaguchi, G., and Goodman, L. S. (1946). *Fed. Proc.*, **5**, 195.

—— and Gump, W. S. (1949). *J. Pharmacol.*, **97**, 25.

Peart, W. S. (1956). *Hypotensive Drugs*, ed. M. Harington, p. 71. London: Pergamon Press.

Brit. J. Pharmacol. (1957), **12**, 498.

THE ANTAGONISM OF THE VASCULAR EFFECTS OF 5-HYDROXYTRYPTAMINE BY BOL 148 AND SODIUM SALICYLATE IN THE HUMAN SUBJECT

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2-Bromo-(+)-lysergic acid diethylamide (BOL 148) is a specific antagonist of the constrictor response of the forearm and hand blood vessels to intra-arterial infusions of 5-hydroxytryptamine. This has been shown to be a direct action in the tissue concerned, since the antagonism was as effective when the drug was given by local arterial injection as when given intravenously. Sodium salicylate is also a specific antagonist of 5-hydroxytryptamine, but its action is indirect, occurring only when the general serum level was raised above 10 or 20 mg.%; local limb concentrations of 30 to 40 mg.% had no antagonistic effect. The mechanism of the antagonistic effect of salicylate is not known. It is not related to the associated hyperventilation but is possibly due to the secondary release of, or synergism with, some other substance, probably a hormone.

A number of substances including lysergic acid derivatives have been shown by Gaddum and Hameed (1954) and other workers to antagonize the action of 5-hydroxytryptamine (5-HT) on isolated smooth muscle preparations. The effect of some of these substances on the response of the blood pressure to intravenous 5-HT has also been studied in animals (Page and McCubbin, 1953; Salmoiraghi, McCubbin, and Page, 1957) and in man (Spies and Stone, 1952).

The vascular responses in the upper limbs of normal human subjects to intra-arterial injections of 5-HT have been described (Roddie, Shepherd, and Whelan, 1955). In the present paper a comparison is made of the antagonistic effect on these responses of 2-bromo-(+)-lysergic acid diethylamide bitartrate (BOL 148) with that of sodium salicylate, which has been shown by Kelemen (1957) to diminish the oedema of the paw of the rat produced by locally injected 5-HT.

MATERIALS AND METHODS

The investigations were carried out on ourselves, our colleagues, and medical students. The subject rested on a couch for at least 30 min. before the first observation was made. The room temperature was kept at a constant level in the range 22 to 25°. An indwelling needle was inserted into the left brachial artery in the antecubital fossa and through it either 0.9% saline or a solution of the drug was infused at a rate of 4 ml./min. using a mechanically driven syringe and a length of polythene tubing. Measurements of forearm blood flow were made by venous occlusion plethysmography using the water-filled, mechanically stirred plethysmographs described by Greenfield (1954), the water temperature being 34 to 35°.

Changes in the circulation through the skin of the finger were followed using the heat flow discs described by Hatfield (1950). These were applied to the finger pulp using Nobecutane (Evans) and the whole hand inserted into a plethysmograph, the temperature of which was carefully maintained at 29°. In most experiments control observations were made on the opposite limb.

5-Hydroxytryptamine creatinine sulphate (Sandoz) was infused intra-arterially for periods of 5 to 10 min. at rates of 1, 4, or 16 μg./min. Solutions were prepared in 0.9% saline so that the dose for 1 min. was contained in 4 ml. The antagonists were administered intravenously into an antecubital vein or intra-arterially by the same needle as the 5-HT. The intravenous doses were 1.5 mg. of 2-bromo-(+)-lysergic acid diethylamide bitartrate in 1 to 2 min. and 3 to 4 g. of sodium salicylate (B.D.H.) in 100 to 150 ml. saline over 3 to 5 min. The intra-arterial doses were 10 μg./min. of BOL 148 and 20 mg./min. of sodium salicylate given for 3 to 10 min. before and the 5 min. during the 5-HT infusion. These arterial doses were calculated to give approximately the same local blood concentration arriving in the forearm as prevailed during the intravenous infusions. In 6 experiments sodium salicylate was given intra-arterially in larger doses of 40 to 200 mg./min. for periods up to 20 min. In some experiments serum salicylate levels were de-

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termined by the method of Keller (1947), and respiratory movements recorded using stethographs around the chest and abdomen (Shepherd, 1951 ; Dornhorst and Leathart, 1952).

Results

Subjective Effects.—During intra-arterial infusion of 5-HT a tingling sensation was experienced in the forearm and hand with doses of 4 and 16, and usually with 1 μg./min. This was reduced or abolished by BOL 148 given either intra-arterially or intravenously and by sodium salicylate given intravenously. No symptoms were noted during administration of BOL 148 by either route, but two subjects complained of tinnitus after an intravenous injection of 4 g. of sodium salicylate. Intra-arterial infusions of sodium salicylate were well tolerated up to a concentration of 50 to 100 mg. in 4 ml. saline/min. ; when the concentration was increased to 100–200 mg., a burning sensation was noted along the course of the brachial artery and its branches in the forearm. In two subjects, intravenous infusion of sodium salicylate in a concentration of 3 to 4 g.% in saline caused pain along the course of the vessel, which ceased abruptly when saline was flushed through. On no occasion did sodium salicylate intra-arterially (12 subjects) or intravenously (12 subjects) cause thrombosis or have any other residual effects on the vessels perfused.

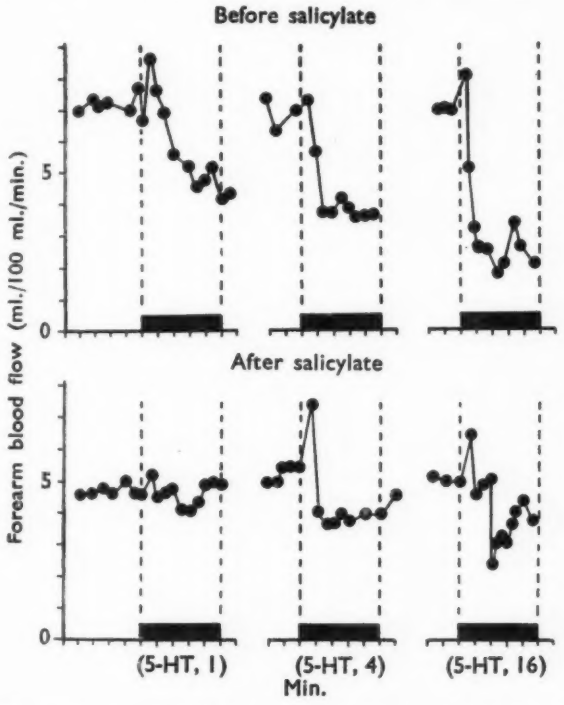

FIG. 1.—The forearm blood-flow changes in response to infusion of 5-HT into the brachial artery before and after 3 g. of sodium salicylate intravenously. The flow is expressed in ml./100 ml./min. The solid rectangles represent the periods of infusion of 5-HT and the numerals in parentheses below them indicate the doses in μg./min. A 10 to 15 min. interval elapsed between each infusion.

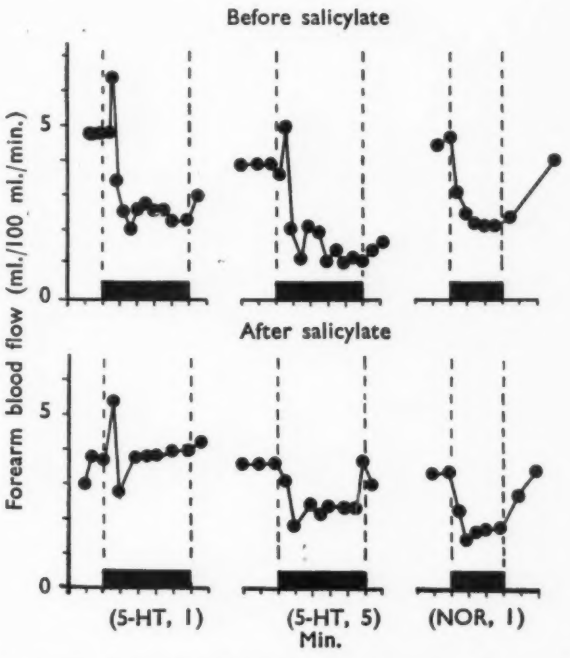

FIG. 2.—The forearm blood flow changes in response to infusions of 5-HT and noradrenaline (NOR) into the brachial artery before and after 3 g. of sodium salicylate intravenously. See Fig. 1 for explanation of symbols.

Forearm Blood Flow.—The changes in forearm blood flow during intra-arterial infusions of 5-HT have already been described (Roddie *et al.*, 1955), and these were confirmed in the present study. At all doses, there was usually an initial transient increase in blood flow lasting for ¼ to 1 min. Subsequently the blood flow fell below the resting level by approximately 30% with 1 μg./min., 50% with 4 μg./min. and 70% with 16 μg./min. (averages of 30 experiments). These levels of flow were reached after 2 to 3 min. of the infusion and persisted as long as it continued. On returning to saline the flow gradually rose to that on the control side over 10 to 20 min. Repeated doses at 10 to 20 min. intervals gave approximately the same response on each occasion.

Intravenous Administration of Antagonists.—Fig. 1 shows the results of a typical experiment in which the forearm blood flow was measured during intra-arterial infusions of 5-HT before and after intravenous administration of 3 g. of sodium salicylate. The initial transient increase in flow

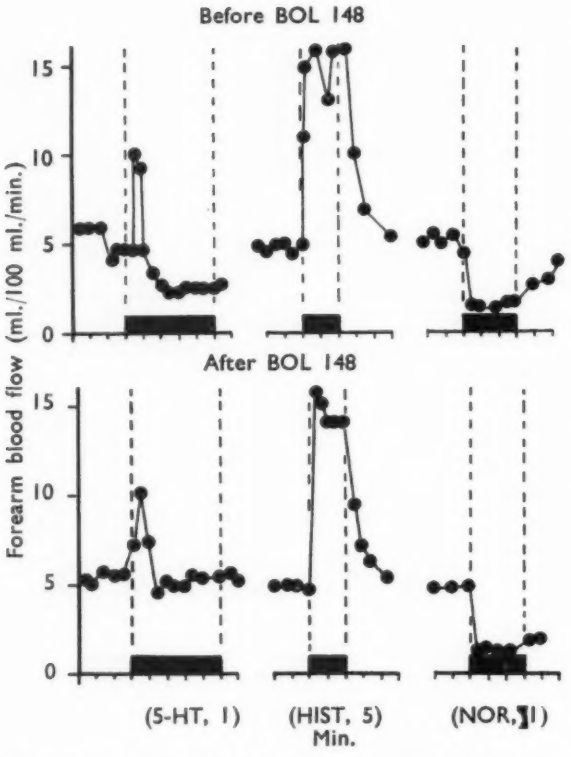

FIG. 3.—The forearm blood flow changes in response to infusions of 5-HT, histamine, and noradrenaline into the brachial artery before and after 1.5 mg. of BOL 148 intravenously. See Fig. 1 for explanation of symbols.

was not consistent in this experiment, but it was not abolished by the sodium salicylate. The fall in flow produced by 1 μg./min. of 5-HT was almost completely abolished, while the responses to 4 μg./min. and 16 μg./min. were considerably reduced. That this antagonistic effect of sodium salicylate was a specific one and not a generalized inhibition of the smooth muscle of the blood vessels is shown by the fact that the response to 5-HT was abolished or reduced by a dose of sodium salicylate which did not affect the response to noradrenaline (Fig. 2). The antagonistic effect of intravenous administration of BOL 148 on the response to 1 μg./min. of 5-HT intra-arterially is shown in Fig. 3. The initial transient dilatation was not affected, but the constrictor response was abolished. The dilator effect of histamine and the constrictor effect of noradrenaline, however, were not altered. The results of all experiments in which the responses to 1, 4, and 16 μg./min. of 5-HT were determined before and after BOL 148 and sodium salicylate intravenously are shown in Fig. 4. The level of forearm blood flow during the last 2 min. of the 5 min. infusion has been expressed as a % of the pre-infusion level corrected for any general fluctuations in flow by reference to the level in the control forearm (Duff, 1952; Greenfield and Patterson, 1954). Each pair of points in each graph represents a separate experiment. It is clear that the two drugs given in this way have an approximately equal antagonistic effect on the responses of the forearm vessels to intra-arterial 5-HT.

In two experiments in which the same dose of 5-HT was given at intervals after the antagonist, the inhibition of the constrictor effect was still present after 1½ to 2 hr.

Intra-arterial Administration of Antagonists.—When BOL 148 was given intra-arterially in a dose of 10 μg./min. for 5 min. before and the 5 min. during the 5-HT infusion, a similar antagonism was exhibited. That this was due to the local, and not the general, effect of the drug is shown by the experiment illustrated in Fig. 5. In this ex-

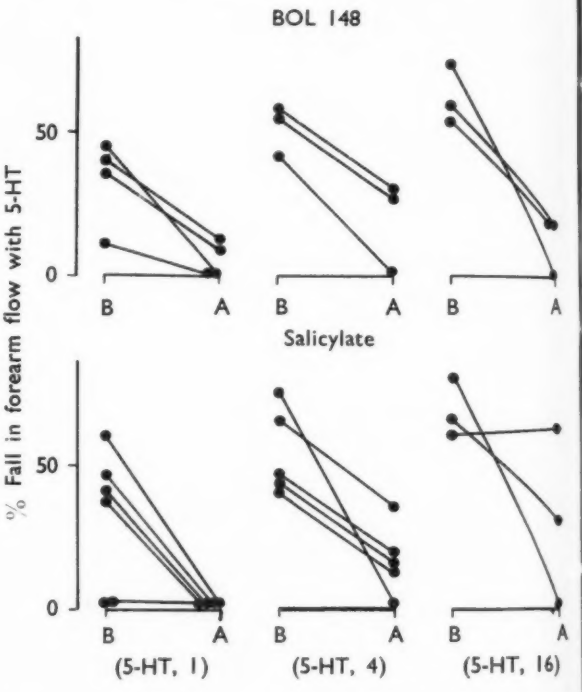

FIG. 4.—The results of all experiments in which the forearm blood flow changes in response to intra-arterial 5-HT were determined before and after 1.5 mg. of BOL 148 intravenously (upper graphs), or 3 to 4 g. of sodium salicylate intravenously (lower graphs). % fall in flow is calculated from the flow in the last 2 min. of the infusion, and the flow that would have been expected if the infusion had not been given. The latter is derived by correcting the pre-infusion level for general fluctuations in vasomotor tone by reference to the control forearm. B, before, and A, after, BOL 148 or sodium salicylate. Each pair of points joined by a line represents one experiment. See Fig. 1 for explanation of symbols.

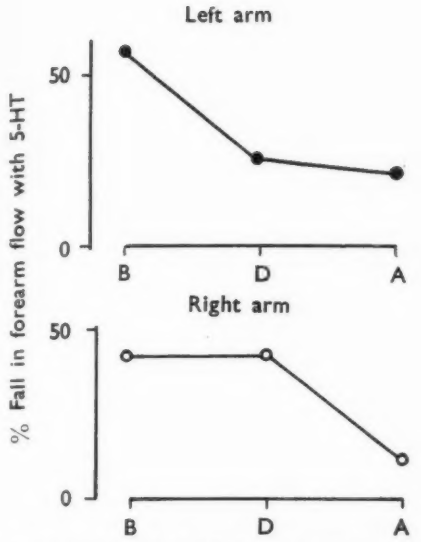

FIG. 5.—The responses of the left (upper graph) and right (lower graph) forearm vessels to the intra-arterial infusions of 5-HT, 4 μg./min., determined simultaneously (B) before, (D) during the infusion of 10 μg./min. of BOL 148 into the left brachial artery, and (A) after 1.5 mg. BOL 148 intravenously. The response is expressed as % fall in flow as in Fig. 4.

artery of each arm, and the responses of the forearm flows to 4 μg./min. of 5-HT measured simultaneously on the two sides. BOL 148 was then given intra-arterially into the left side at the rate of 10 μg./min. for 10 min., and during the second 5 min. the bilateral 5-HT infusion was repeated. The response on the left side was greatly reduced, but the right side responded as before. When, however, 1.5 mg. of BOL 148 was given intravenously and the bilateral infusions of 5-HT again repeated, the response on the right side was reduced to approximately the same level as the left.

Sodium salicylate was only effective intra-arterially when given in doses which caused the general blood level to approach that achieved in the intravenous experiments. Doses as high as 100 mg./min. for 10 min. had no effect on the response to 5-HT. In the experiment illustrated in Fig. 6, the response to 4 μg./min. 5-HT was recorded. Sodium salicylate was then given intra-arterially at the rate of 20 mg./min. for 6 min. and for the last 5 min. the 5-HT infusion was repeated. The response was unchanged even though the venous blood returning from the arm contained 40 mg./100 ml. of sodium salicylate. At this time the general blood level was 2.5 mg./100 ml., but when it was raised to 33 mg./100 ml. by the intravenous administration of 4 g. of sodium salicylate the response to a further 4 μg./min. of 5-HT was greatly reduced. The results of this and 6 similar experiments on different subjects are shown in Fig. 7. In one subject the constrictor response to 5-HT was not affected either by intra-arterial or by intravenous sodium salicylate. In the others the response was reduced or abolished only when the sodium salicylate was given intravenously. These findings suggest that the antagonistic effect of sodium salicylate is not a local one on the tissues but due to some indirect action of the drug.

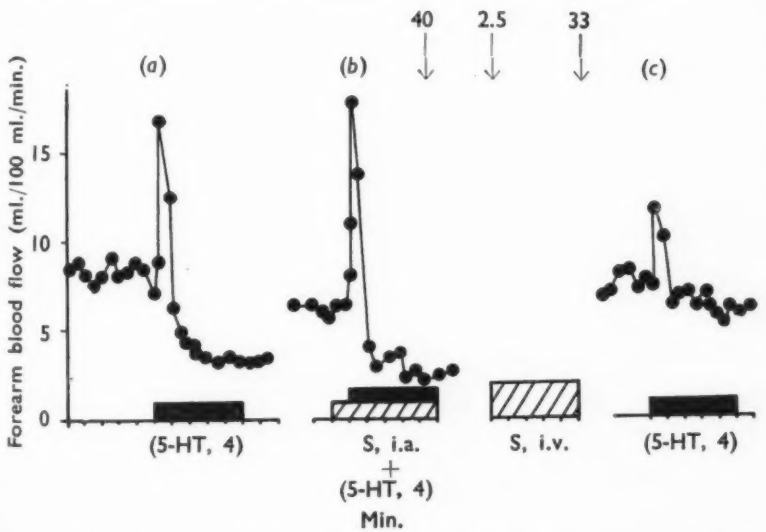

FIG. 6.—The changes in the left forearm flow (ml./100 ml./min.) in response to the intra-arterial infusion of 4 μg./min. of 5-HT (*a*) before, (*b*) during the infusion of 20 mg./min. of sodium salicylate into the same brachial artery (S, i.a.), and (*c*) after 4 g. sodium salicylate intravenously (S, i.v.). The arrows represent the time at which blood samples were taken from a left antecubital vein, and the numerals the serum sodium salicylate content in mg.%.

Hand Blood Flow.—Changes in hand blood flow were followed by measurement of heat flow from the skin of the pulp of the finger. The plethysmographic method was not used because of the difficulty in interpretation of hand inflow curves as a consequence of reduction in the distensibility of the hand vessels (Roddie *et al.*, 1955 ; Glover, Greenfield, Kidd, and Whelan, 1957). In 4 experiments, the level of heat flow was determined during intra-arterial infusions of 5-HT both before and after intravenous injection of 2 g. of sodium salicylate. 5-HT caused a fall in heat elimination and this constrictor response

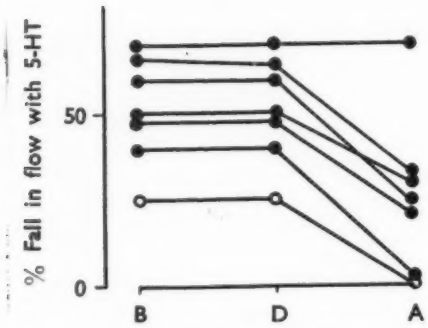

FIG. 7.—The results of seven experiments of the type illustrated in Fig. 6. The response to 5-HT intra-arterially is expressed as a % fall in flow. Solid circles represent a dose of 4 μg./min. of 5-HT; open circles a dose of 1 μg./min. of 5-HT. B, before administration of sodium salicylate. D, during the intra-arterial infusion of 20 mg./min. of sodium salicylate. A, after the intravenous injection of 3 to 4 g. of sodium salicylate.

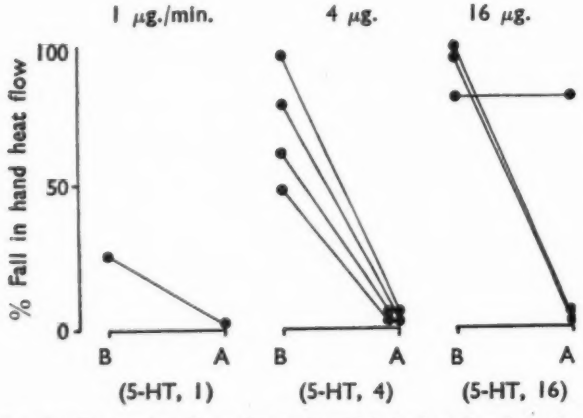

FIG. 8.—The results of experiments in which the heat flow from the finger skin during intra-arterial infusions of 1, 4, or 16 μg./min. of 5-HT was determined before (B) and after (A) 2 g. sodium salicylate intravenously. The response is expressed as % fall in heat elimination and is calculated as in Fig. 4.

was abolished by sodium salicylate on all but one occasion, when the response to 16 μg./min. was unaffected (Fig. 8). It is concluded that the vessels of the hand respond to 5-HT and to sodium salicylate in the same way as do those of the forearm.

DISCUSSION

Lysergic acid and its derivatives including 2-bromo-(+)-lysergic acid diethylamide bitartrate have been shown to antagonize the effects of 5-HT on isolated smooth muscle preparations (Gaddum and Hameed, 1954), but these substances have been found to be without effect on the symptoms of patients suffering from carcinoid tumour (Snow, Lennard-Jones, Curzon, and Stacey, 1955). The results of the present experiments demonstrate that BOL 148 is an effective antagonist of certain of the vascular responses to 5-HT in the limbs of normal human subjects. The antagonism is displayed whether the BOL 148 is given intravenously, or locally by intra-arterial injection into the limb segment concerned. It is concluded that the antagonistic action is dependent upon a direct effect of BOL 148 at the site of action of the 5-HT, namely, the smooth muscle of the blood vessels. The antagonism is not a consequence of a non-specific inhibition of smooth muscle, because the dilator response of the vessels of the part to histamine and the constrictor response to noradrenaline can be shown to be unaffected at a time when the response to 5-HT has been abolished or considerably diminished by BOL 148.

The antagonistic action of sodium salicylate on the vascular responses of the forearm vessels to 5-HT is likewise a specific one, but, in contrast to that of BOL 148, it appears to be an indirect action. Introduction of sodium salicylate locally into the limb is only effective if given in amounts large enough to circulate and to raise the serum level to 20 to 25 mg.% or more. Smaller doses, though providing a high local blood concentration, were without effect on the responses to 5-HT. In the intra-arterial experiments care was taken to achieve as far as possible the same local conditions in the forearm as during the intravenous experiments, by maintaining the sodium salicylate level in the perfusing blood at approximately the same level and by administering it for the same period before and during the 5-HT infusion.

The present experiments provide no explanation of the mechanism of the indirect antagonistic effect of sodium salicylate. Sodium salicylate given intravenously has been shown to stimulate respiration and thus cause a rise in blood *p*H (Graham and Parker, 1948). That this effect of sodium salicylate is unlikely to be the cause of its antagonism to 5-HT is shown by the result of the following experiment. In a normal subject, the respiratory movements were recorded on a smoked drum from stethographs applied around the chest and abdomen, and the responses of the forearm vessels to intra-arterial 5-HT were determined. When an intravenous injection of 4 g. of sodium salicylate was given both the rate and depth of respiration were increased for a period of 10 to 15 min. Towards the end of this time, and while the respiration was still increased, the doses of 5-HT were repeated and the responses were found to be reduced by the usual amount. On a subsequent occasion the same subject hyperventilated for the same period of time at a rate and

depth somewhat greater than before, as judged by "playing back" to him the previous record (Dornhorst and Whelan, 1953; Barcroft, Gaskell, Shepherd, and Whelan, 1954). The responses of the forearm vessels to the 5-HT infusions were the same during as before the period of hyperventilation, showing that the effects of hyperventilation of a degree greater than that due to sodium salicylate provoked no antagonism of the response to 5-HT.

It is possible that sodium salicylate or one of its derivatives releases, activates or combines with some other substance already present in the body but not in the forearm, such as the secretion of an endocrine gland. It has long been recognized that sodium salicylate has a cortisone-like effect, but it has been shown that in man this is not due to an increase in the plasma level of adrenal cortical hormone (Bayliss and Steinbeck, 1954). Kelemen (1957) has shown that salicylate antagonizes the oedema of the paw in the rat produced by locally injected 5-HT in the intact but not in the adrenalectomized animal. This suggests that salicylates depend in some way on the adrenal corticoids for some of their actions, and it seems likely that a similar synergism plays a part in the antagonism of the vascular effects of 5-HT in man.

We are grateful to Sandoz Ltd. for generous supplies of 5-hydroxytryptamine creatinine sulphate and of 2-bromo-(+)-lysergic acid diethylamide.

References

Barcroft, H., Gaskell, P., Shepherd, J. T., and Whelan, R. F. (1954). *J. Physiol.*, **123**, 443.

Bayliss, R. I. S., and Steinbeck, A. W. (1954). *Lancet*, **1**, 1010.

Dornhorst, A. C., and Leathart, G. L. (1952). Ibid., **2**, 109.

—— and Whelan, R. F. (1953). *J. Physiol.*, **119**, 9P.

Duff, R. S. (1952). Ibid., **117**, 415.

Gaddum, J. H., and Hameed, K. A. (1954). *Brit. J. Pharmacol.*, **9**, 240.

Glover, W. E., Greenfield, A. D. M., Kidd, B. S. L., and Whelan, R. F. (1957). *J. Physiol.*, **137**, 16P.

Graham, J. D. P., and Parker, W. A. (1948). *Quart. J. Med.*, **17**, 153.

Greenfield, A. D. M. (1954). *J. Physiol.*, **123**, 62P.

—— and Patterson, G. C. (1954). Ibid., **125**, 508.

Hatfield, H. S. (1950). Ibid., **111**, 10P.

Kelemen, E. (1957). *Brit. J. Pharmacol.*, **12**, 28.

Keller, W. J. (1947). *Amer. J. clin. Path.*, **17**, 415.

Page, I. H., and McCubbin, J. W. (1953). *Amer. J. Physiol.*, **174**, 436.

Roddie, I. C., Shepherd, J. T., and Whelan, R. F. (1955). *Brit. J. Pharmacol.*, **10**, 445.

Salmoiraghi, G. C., McCubbin, J. W., and Page, I. H. (1957). *J. Pharmacol.*, **119**, 240.

Shepherd, J. T. (1951). *Brit. med. J.*, **2**, 1007.

Snow, P. J. D., Lennard-Jones, J. E., Curzon, G., and Stacey, R. S. (1955). *Lancet*, **2**, 1004.

Spies, T. D., and Stone, R. E. (1952). *J. Amer. med. Ass.*, **150**, 1599.

Brit. J. Pharmacol. (1957), **12**, 504.

THE EFFECTS OF ANTICHOLINESTERASES ON THE BRONCHIOLES AND PULMONARY BLOOD VESSELS IN ISOLATED PERFUSED LUNGS OF THE DOG

BY

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The effects of two potent inhibitors of cholinesterase, *iso*propylmethylphosphonofluoridate (sarin) and ethyl pyrophosphate (TEPP), were investigated on the bronchioles and pulmonary blood vessels in isolated dog lung preparations perfused with heparinized blood and ventilated artificially. These anticholinesterases caused gradual bronchoconstriction as indicated by a diminution in tidal air volume, a reduction in compliance and an increase in expiratory resistance. These effects were antagonized by atropine. An increase in pulmonary vascular resistance occurred which was enhanced by the addition of small quantities of acetylcholine to the perfusing blood. The pulmonary vascular effects of acetylcholine, sarin and TEPP were prevented and abolished by atropine. Evidence is presented which suggests that the site of action of these anticholinesterases is, at least in part, peripheral to the autonomic ganglia.

The effects of anticholinesterases on the pulmonary circulation in the dog have been studied recently by Daly (1957) and by Daly and Wright (1957). Under conditions in which respiration, pulmonary blood flow and left atrial pressure were controlled to exclude passive effects on the pulmonary circulation, the intravenous administration of anticholinesterases caused an increase in pulmonary vascular resistance (Daly and Wright, 1957). It was suggested that several mechanisms probably contributed to this response, and evidence was presented that one of these was an alteration in alveolar gas tensions. Owing to the difficulty of controlling other variables it was not possible in these experiments to obtain evidence of a possible direct action of anticholinesterases on the pulmonary blood vessels. This problem has now been investigated and the results are presented in this paper. For the most part, *iso*propylmethylphosphonofluoridate (sarin) was used, but a few experiments were made with ethyl pyrophosphate (TEPP).

METHODS

Dogs, varying in weight from 6 to 14.7 kg., were given morphine hydrochloride (1 to 2 mg./kg. body weight, subcutaneously). Under local anaesthesia, a femoral artery was cannulated, and after intravenous injection of heparin ("Liquemin," Roche Products, 7 to 8 mg./kg.) to prevent intravascular clotting in later stages of making the preparation, the animal was bled to death. By means of a Dale-Schuster pump, the lungs were perfused *in situ* through the pulmonary artery at a constant blood volume inflow. Blood from the cannulated left atrium was collected in a reservoir, the bottom of which was connected to the input side of the pump. To prevent blood escaping from the pulmonary circulation by way of the bronchial vascular system, the superior and inferior venae cavae, the vena azygos, the aorta just above the level of the diaphragm and the oesophagus above and below the lung hilus were ligated. The ventricles were compressed by tying them tightly with tape just below the atrio-ventricular junction.

Both the pump and reservoir were placed in a thermostatically controlled water-bath at 37°. The pulmonary arterial pressure was measured with a small Marey tambour whose displacement was 0.25 ml./cm. saline change in pressure. Changes in the volume of blood in the reservoir were recorded by means of a piston recorder; these indicate inverse changes in lung blood volume provided allowances are made for capacity changes in the manometer (I. de B. Daly, 1928).

In other experiments the lungs were perfused at constant head of pressure. A side-arm in the pulmonary arterial tubing was connected to a small reservoir the blood level in which was maintained constant by an overflow pipe which returned the excess of blood back to the main reservoir. The output of the pump was initially set so that a small

volume of blood overflowed from the small reservoir. The level of the blood in this reservoir varied between 15 and 23 cm. above the pulmonary artery in different experiments. The left atrial blood outflow was measured by means of a recorder similar to that described by Gaddum (1939).

The volume of blood in the reservoir, pump and connecting tubes was 300 to 450 ml.

Lung Ventilation.—In some experiments the lungs were ventilated artificially by negative pressure ventilation. The lungs were enclosed in a reasonably airtight chamber from which the air was continuously exhausted by means of an electric vacuum pump. The negative pressure thereby created was then practically abolished by means of a cam-operated valve admitting atmospheric air to the chamber 12 times/min. The tidal air volume was measured with a small spirometer.

In other experiments the lungs were ventilated at a constant peak inflationary pressure which varied between 5 and 12.5 cm. water in different experiments (Konzett and Rössler, 1940). The ventilation overflow volume, that is the volume of air not entering the lungs but spilling over the constant pressure device, was measured by means of a piston recorder. Since anticholinesterases usually produced large reductions in tidal air volume, it was found more convenient to collect the ventilation overflow volume continuously in a balanced 5-litre spirometer. Thus, an increase in slope of the spirometer trace indicates a diminution in tidal air volume. The ventilation overflow volume (V.O.V.)/respiratory cycle can be determined at any instant from the slope of the trace and the speed of the respiratory pump:

$$\text{V.O.V. (ml./respiration cycle)} = \frac{\text{V.O.V. (ml./min.)}}{\text{Respiratory pump rate (rev./min.)}}$$

The tidal air volume can then be calculated by subtracting the ventilation overflow volume from the corrected stroke volume of the respiratory pump. The stroke volume of the pump was measured at atmospheric pressure and was corrected for the pressure at which the lungs were inflated, taking into account the volume of air in the pump at the beginning of its stroke and in the connecting tubes between the pump and the lungs. This latter volume was approximately 800 ml. A change of 3 ml. in ventilation overflow volume/respiratory cycle is detectable by this method.

In a few experiments the compliance and expiratory resistance of the lungs were measured by a modification of the method used by Dixon and Brodie (1903) and by Comroe, Nisell and Nims (1954). The lungs were ventilated by negative pressure as described above and the tidal air volume was recorded on a fast-moving paper by means of a spirometer. On inspiration a volume plateau was reached, and then, by allowing the lungs to collapse passively by means of their own elastic recoil, the expiratory volume curve was obtained. The respiratory rate which was determined by the expiratory valve was 12 cycles/min., and this was found to be too fast when bronchial obstruction became severe, resulting in expiration occurring before the inspiratory volume plateau was reached. It was more convenient therefore to operate the valve by hand during the test periods.

In all preparations complete collapse of the lungs during expiration was prevented by immersing a tube connecting the expiratory side of the Starling respiration pump 2 to 3 cm. under water; in the case of lungs ventilated by negative pressure, the extrapulmonary pressure during expiration varied from -1.5 to -3 cm. water in different experiments.

In three preparations the lungs were not ventilated; the changes in intrapulmonary volume were measured at ambient pressure by means of a small volume recorder connected directly to the trachea.

A 1:1000 solution of *iso*propylmethylphosphonofluoridate (sarin) or a 1:100 solution of ethyl pyrophosphate (TEPP) was usually injected into the pulmonary arterial inflow tubing. Other drugs used were: acetylcholine (Roche), atropine sulphate (B.D.H.), hexamethonium bromide ("Vegolysen," May & Baker), adrenaline chloride, 0.1% solution with 0.5% chloretone (Parke, Davis), noradrenaline ("Levophed," Bayer Products) and isoprenaline (Burroughs Wellcome).

Results

In the experiments to be described, the range of single doses of sarin injected into the pulmonary arterial tubing or into the venous reservoir was 90 to 500 μg., and for TEPP 3 to 5 mg. No differences in the responses to these two anticholinesterases were found and the results with each of these compounds will therefore be presented together.

The Effect of Sarin and TEPP on Tidal Air Volume

The effect of these anticholinesterases on the tidal air volume was tested in 14 experiments in which artificial respiration was carried out by positive or negative pressure ventilation. The results are summarized in Table I. In 11 of the experiments the initial dose of sarin or TEPP caused a mean reduction in tidal air volume of 55.4% (range 2 to 100%). Such an effect is illustrated by Fig. 1 and is indicated by the increase in the slope of the ventilation overflow volume trace. In experiment Nos. 1 and 18 (Table I) the injection of the anticholinesterase was made during an infusion of acetylcholine. In three experiments there was no effect (Table I).

In one additional experiment (No. 14 of Table I) the lungs were ventilated at constant volume instead of at constant pressure, and TEPP caused an increase in peak intrapulmonary pressure from 12 to 23 cm. water (Fig. 2).

TABLE I

THE EFFECTS OF SARIN AND OF TEPP ON ISOLATED LUNGS PERFUSED AT CONSTANT BLOOD VOLUME INFLOW OR AT CONSTANT HEAD OF PRESSURE. THE LUNGS WERE VENTILATED WITH EITHER CONSTANT POSITIVE OR NEGATIVE PRESSURE

S=Sarin. T=TEPP. * Intrapulmonary pressure increased from 12 to 23 cm. water when ventilation was carried out with an intermittent constant volume of air.

Expt. No.	Drug	Tidal Air Volume % Change	Pulmonary Arterial Pressure (cm. Saline)		Left Atrial Outflow (ml./min.)		Vascular Resistance % Change	Remarks
			Before	After	Before	After		
1	S	−94			240	220	+8	Infusion of acetylcholine (20 μg./min.)
2	S	0			645	645	0	
3	S	No ventilation			325	325	0	
4	S	,,			415	415	0	Hexamethonium
5	S	,,			420	420	0	,,
6	S	−3			425	425	0	
7	S	0	14	15			+7	
8	S	−62	21	22·5			+7	
9	S	−7	14	14			0	
10	S	−33	6·5	6·5			0	
11	S	0	13	13			0	Hexamethonium
12	S	−2	8	8			0	,,
13	T	−60	12·5	14			+12	,,
14	T	*	18	20·5			+14	
15	T	−81	18·5	18·5			0	Injection of acetylcholine (160 μg.)
16	S	−100	17	18·5			+9	
17	T	−96	16	19			+19	
18	T	−71	12	15·5			+29	Infusion of acetylcholine (20 μg./min.)

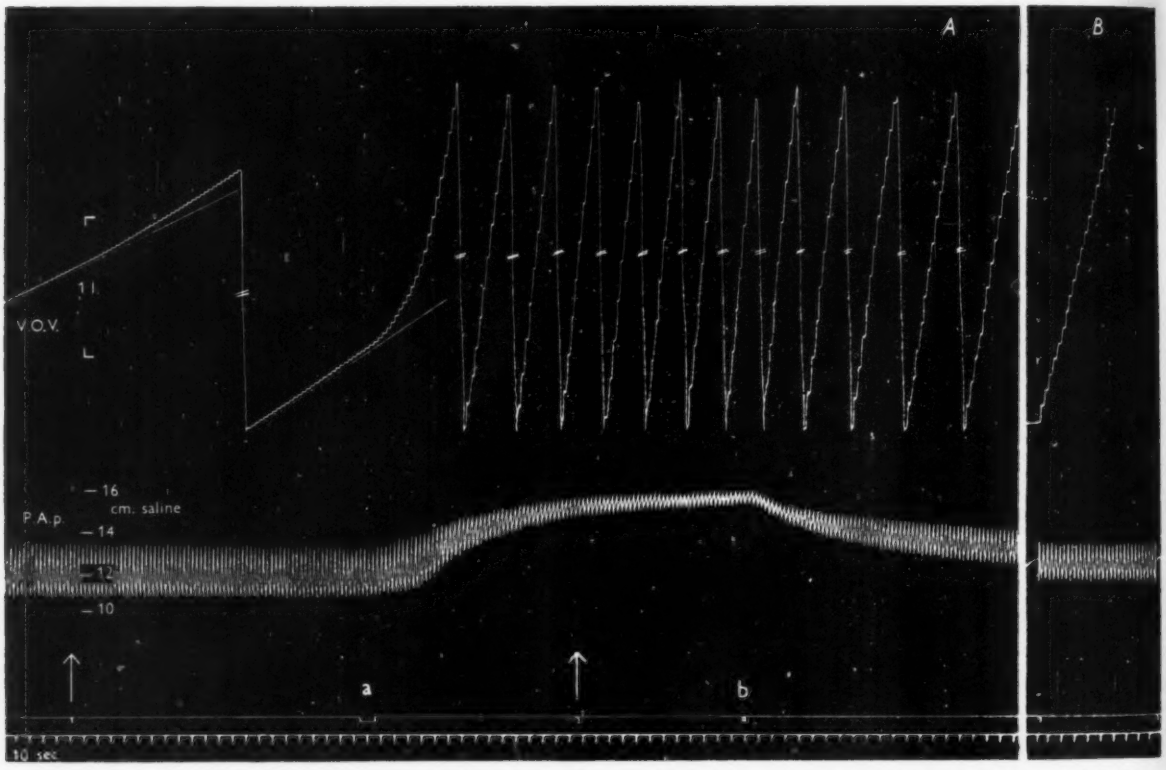

FIG. 1.—Dog, male, 14.0 kg. Isolated lungs perfused at constant blood volume inflow. Lungs ventilated at constant peak inflationary pressure. Respiratory pressure, 10 cm. water. Respiration pump stroke, 410 ml. An infusion of acetylcholine, 20 μg./min., was given into the pulmonary arterial inflow tubing between the arrows. At a, TEPP, 3 mg., and at b, atropine, 1 mg., were given. Between *A* and *B* there was an interval of 6 min. during which a further dose of atropine, 1 mg., was given. V.O.V.=ventilation overflow volume; P.A.p.=pulmonary arterial pressure.

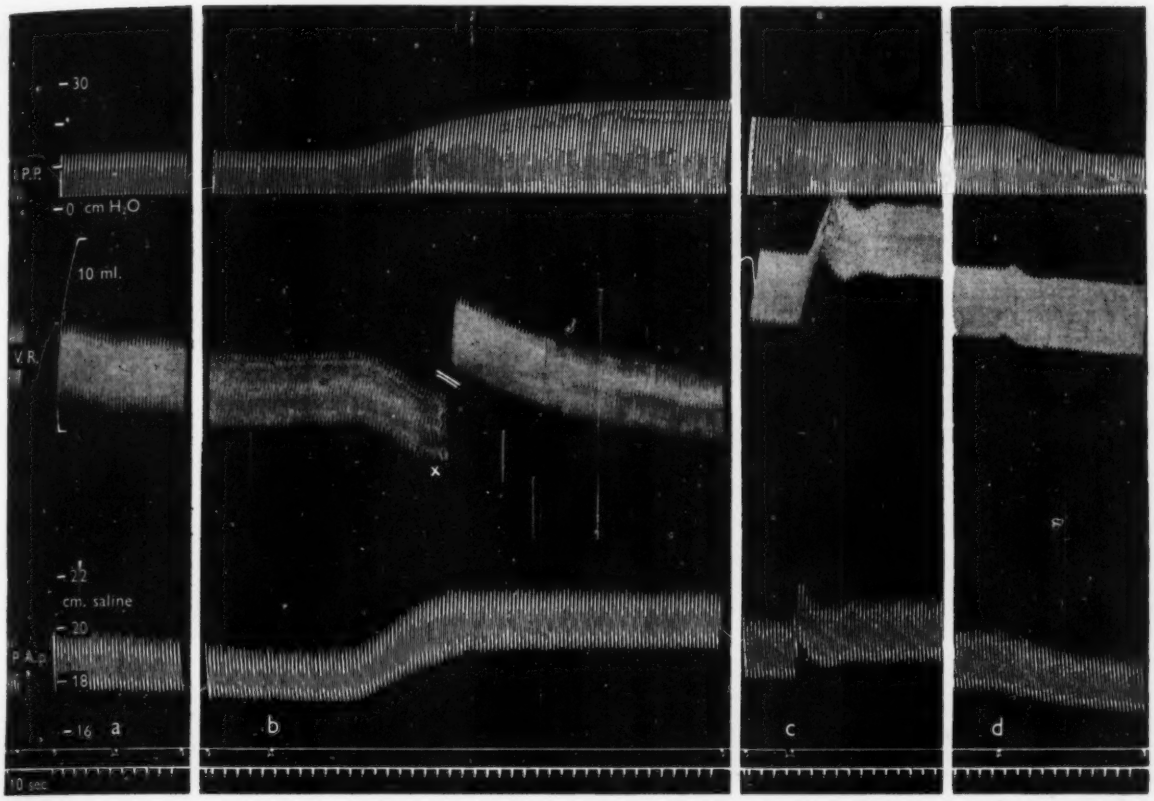

FIG. 2.—Dog, male, 11.0 kg. Isolated lungs perfused at constant blood volume inflow. Positive pressure ventilation with a Starling "Ideal" pump (constant volume). Records from above downwards: intrapulmonary pressure (I.P.P.); changes in volume of the venous reservoir (V.R.) in which an upward trend of the trace indicates an increase in volume of blood in reservoir; and pulmonary arterial perfusion pressure (P.A.p.). a, 0.2 ml. sodium chloride solution, 0.9% w/v; b, TEPP, 5 mg.; c, adrenaline, 10 μg.; d, atropine, 1 mg. All injections were made into the pulmonary arterial tubing. The downward excursion of the recorder was limited at X.

The response of the tidal air volume differed considerably from that observed in the entire animal using comparable methods of artificial respiration (Daly, 1957). In isolated lungs there was a longer latency before the effect came on, and the full response usually took several minutes to develop. A similar effect was observed by Alcock, Berry and I. de B. Daly (1935) in isolated perfused dog lungs in response to eserine. In the present experiments, however, it was found that the effect of the anticholinesterase appeared more quickly if it was given either immediately after an injection of acetylcholine or during a continuous infusion of acetylcholine. It had been found previously that the acetylcholine contraction of the isolated tracheal muscle of the guinea-pig was potentiated by sarin and by TEPP (de Candole, Douglas, Evans, Holmes, Spencer, Torrance and Wilson, 1953). In the present experiments acetylcholine in doses which were just effective in the untreated preparation caused a considerable reduction in tidal air volume after sarin or TEPP.

Three experiments were carried out in which the compliance and resistance of the lungs were measured. Both sarin and TEPP caused a reduction in compliance and an increase in inspiratory and expiratory resistances. Although the static pressure-volume relationship of the lungs was determined before the injection of the anticholinesterase, it was not possible to repeat the measurements after injection owing to the rapidity of the change in the visco-elastic properties of the lungs. Thus no accurate values can be given for the changes in expiratory resistance which depend upon knowing this relationship. It is clear, however, from Fig. 3 that a considerable obstruction to expiration occurred.

In order to gain some information on the site of action of the anticholinesterases, three preparations (expt. Nos. 11, 12, and 13 of Table I) were pretreated with hexamethonium to block intrapulmonary autonomic ganglia. Hexamethonium itself, in doses of 5 to 100 mg., had no effect on the tidal air volume. In two of the experiments,

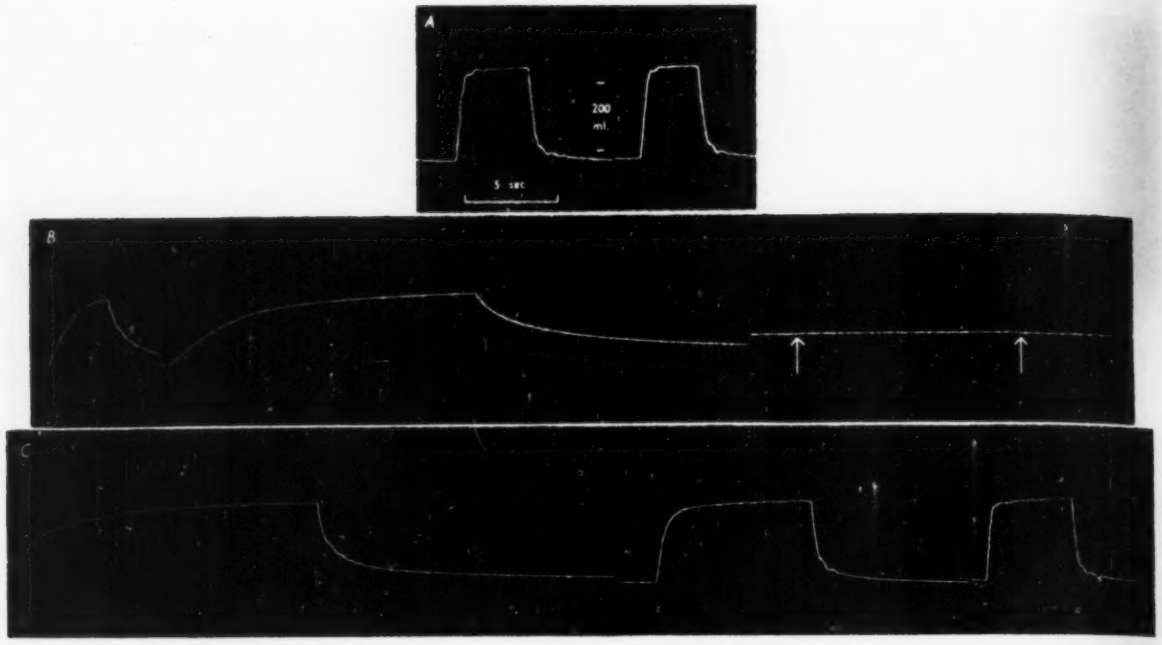

FIG. 3.—Dog, male, 10.3 kg. Isolated lungs perfused at constant head of pressure (21 cm. saline). Negative pressure ventilation. Inspiratory pressure, −15 cm. H_2O; expiratory pressure, −1.5 cm. H_2O. Record is that of the tidal air volume (inspiration upwards) showing changes in compliance and resistance of the lungs. *A*, control. *B* shows three respiratory cycles after injection of sarin, 600 μg., into the pulmonary arterial tubing. In the third cycle the negative inspiratory pressure applied to the outside of the lungs (between the arrows) caused no change in tidal air volume. *C*, effect of atropine, 1 mg., which was injected immediately after the tidal air volume had been reduced to zero as a result of injection of sarin.

sarin or TEPP injected subsequently still caused a reduction in tidal air volume; in the other, sarin had no effect. In one further experiment in which hexamethonium was injected at the height of the response produced by sarin, the tidal air volume remained unaffected. These results indicate that the site of action of these anticholinesterases is peripheral to the ganglia.

Effect of Adrenaline, Noradrenaline, and Isoprenaline.—In a few experiments, these drugs were injected into the pulmonary arterial tubing to test their efficacy in relieving bronchospasm produced by anticholinesterases. In three of four experiments, adrenaline 5 to 25 μg. and noradrenaline 2 to 20 μg. caused a small increase in tidal air volume or a decrease in intrapulmonary pressure after poisoning with sarin or TEPP (Fig. 2c); in the fourth experiment, they had no effect. On the other hand, isoprenaline 3 to 30 μg. invariably caused increases in tidal air which were larger than those produced by either adrenaline or noradrenaline when equal doses of the drugs were administered. Owing to the variability of the responses to repeated injections of the same drug, however, it was not possible to arrive at an accurate value for their relative antispasmodic potencies. In this connexion, Hebb and Konzett (1949) found that a dose of isoprenaline only one-tenth that of adrenaline was usually required to produce identical responses on the tidal air volume.

Effect of Atropine.—In doses of 1 to 10 mg., atropine reversed the effects of sarin and TEPP in 14 of 15 experiments, although in only six of these did the tidal air volume or intrapulmonary pressure return to its original value. In one experiment atropine was without effect. These responses are illustrated by Figs. 1 (at b) and 2d. In the experiments shown in Figs. 3*C* and 4*B* the expiratory resistance returned approximately to normal, although the compliance remained reduced. In experiments in which after poisoning with an anticholinesterase atropine failed to increase the tidal air volume to its original value, it was found that the most effective way of restoring it was to hyperinflate the lungs once or twice by an increased positive pressure in the trachea (Fig. 4*C*) or by an increased negative pressure applied to the outside of the lungs.

Subsequent injections of acetylcholine, sarin or TEPP in five atropinized preparations were without effect on the tidal air volume. In one preparation pretreated with atropine (2.5 mg.) neither sarin nor acetylcholine had any effect.

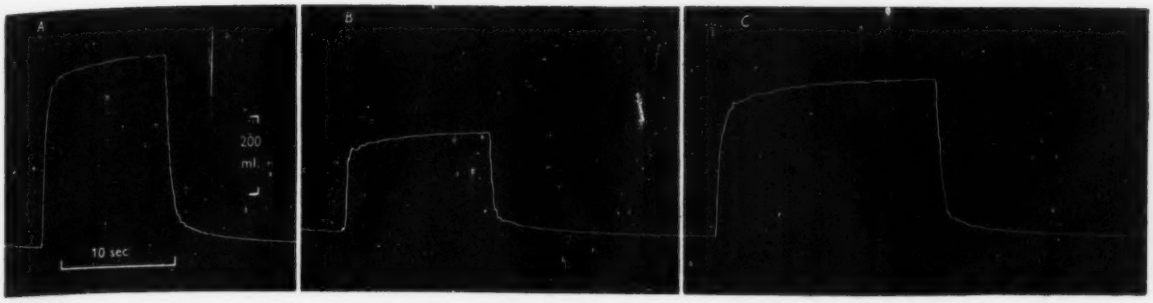

FIG. 4.—Dog, male, 14.0 kg. Isolated lungs perfused at constant blood volume inflow. Negative pressure ventilation. Inspiratory pressure, −10 cm. water; expiratory pressure, −1.5 cm. water. Record of tidal air volume. *A*, control. TEPP 3 mg. was then injected which resulted in a reduction in tidal air volume to 115 ml., and was followed by 2 mg. atropine. *B* shows maximum recovery of the tidal air after atropine. *C* was taken using the same respiratory pressure but after the lungs had been inflated with an increased negative pressure.

Pulmonary Vascular Effects of Sarin and TEPP

The effects of the initial dose of sarin and TEPP upon the pulmonary vascular resistance are shown in Table I. An increase in vascular resistance occurred in eight experiments as indicated by an increase in pulmonary arterial pressure when the lungs were perfused at constant blood volume inflow or by a decrease in left atrial outflow at constant head of pressure. In 10 experiments there was no effect.

In 4 of 7 experiments, changes in the volume of blood in the reservoir were observed on injection of anticholinesterases, but they were small and may have been due to capacity changes in the manometer rather than to alterations in lung blood volume.

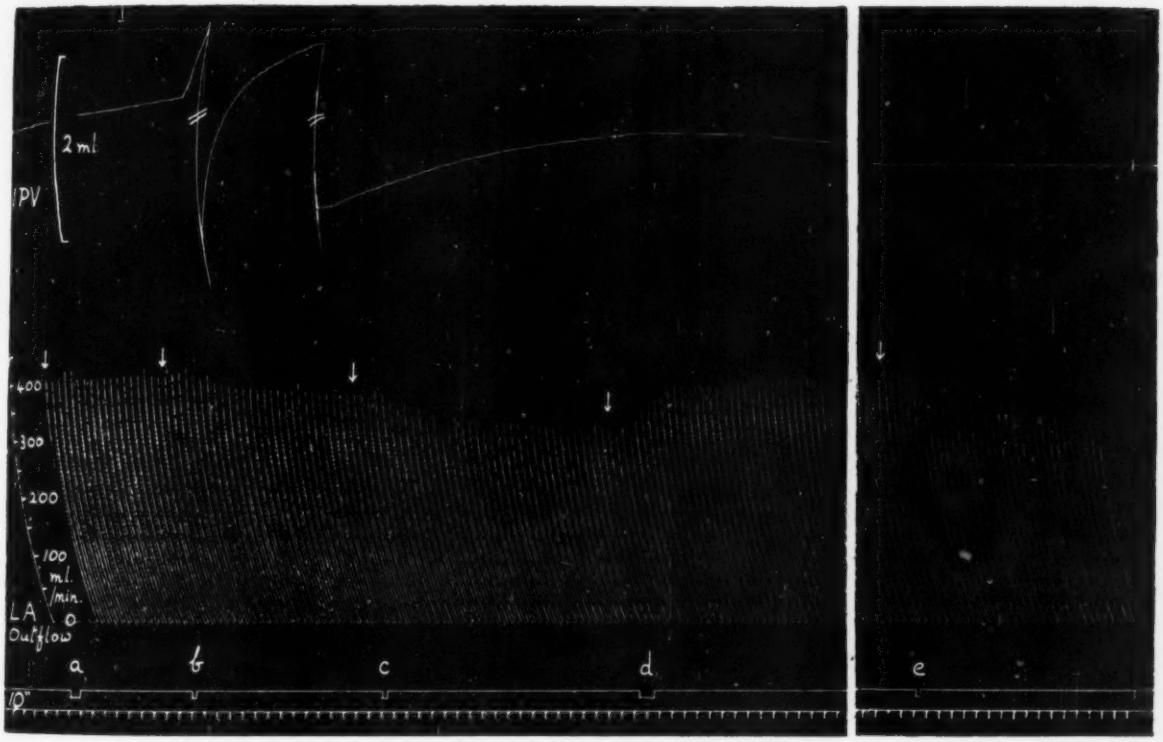

FIG. 5.—Dog, male, 10.2 kg. Isolated lungs perfused at constant head of pressure. No pulmonary ventilation. The upper record shows the *changes* in intrapulmonary air volume (I.P.V.) measured by a small volume recorder connected to the tracheal cannula (an upward trend of lever denotes decrease in intrapulmonary air volume). The lower record is of the left atrial outflow (LA outflow). Hexamethonium, 100 mg. before recording began. At a, sarin, 325 μg. At b and c, acetylcholine, 40 μg. At d, atropine 5 mg. In e, adrenaline, 50 μg.

It was found that anticholinesterases produced larger changes in pulmonary vascular resistance if they were injected on a " background " of acetylcholine. For instance, in experiment Nos. 1 and 18 of Table I an infusion of 20 μg./min. acetylcholine was begun about 3 min. before injecting sarin or TEPP and was continued for 3 min. afterwards. This rate of infusion was not sufficient in itself to alter the pulmonary vascular resistance although it did cause a slight reduction in tidal air volume in both cases. The injection of 360 μg. sarin during the infusion caused a fall in left atrial outflow from 240 to 220 ml./min. (8%); in the second experiment 3 mg. TEPP caused a rise in pulmonary arterial pressure from 12 to 15.5 cm. saline (29%). This effect is illustrated by Fig. 1. A second dose of sarin or TEPP nearly always caused a further increase in pulmonary vascular resistance. These responses were observed in preparations ventilated by positive or negative pressure ventilation and in those in which no ventilation was carried out. Furthermore, they occurred in preparations pretreated with hexamethonium (5 to 100 mg.).

In three normal preparations the smallest effective dose of acetylcholine caused an increase in pulmonary vascular resistance and this effect was potentiated by sarin or TEPP. Reductions in left atrial outflow up to 30% were observed on injection of acetylcholine in preparations treated with these anticholinesterases. The typical effect is shown in Fig. 5b and c. An increase in volume of blood in the reservoir also occurred, indicating a diminution in pulmonary blood volume. A similar effect was observed by Alcock *et al.* (1935).

Effect of Adrenaline, Noradrenaline, and Isoprenaline.—After injection of sarin or TEPP, adrenaline, 5 to 25 μg., and noradrenaline, 2 to 20 μg., caused an increase in pulmonary vascular resistance and an increase in volume of the blood in the reservoir, indicating a diminution in lung blood volume. These effects are shown in Figs. 2c and 5e. On the other hand, isoprenaline, 3 to 30 μg., invariably caused a small but definite decrease in pulmonary vascular resistance. In confirmation of the results of Hebb and Konzett (1949), this drug also produced a fall in pulmonary vascular resistance in the normal isolated perfused lung preparation.

Effect of Atropine.—In doses varying from 1 to 10 mg., atropine almost invariably reversed the pulmonary vasopressor response of sarin and TEPP. These effects are illustrated by Figs 1b, 2d, and 5d. Further doses of these anticholinesterases or of acetylcholine injected after atropine were without effect. In one preparation pretreated with atropine (2.5 mg.), neither sarin, total dose 870 μg., nor acetylcholine had any effect on the pulmonary arterial pressure.

Discussion

Bronchomotor Effects.—Our experiments have shown that both sarin and TEPP cause bronchoconstriction as indicated by a diminution in compliance of the lungs and an increase in expiratory resistance. These results are in keeping with those obtained by other workers using bronchoconstrictor drugs such as acetylcholine and pilocarpine (Bayliss and Robertson, 1939; Mount, 1956).

Anticholinesterases have been shown previously to cause bronchoconstriction in isolated perfused lungs (Alcock *et al.*, 1935; Green, McKay, and Krop, 1947: Bhattacharya and Pochet, 1956). They also cause contraction of muscle in isolated trachea or bronchial chain preparations (Macht and Ting, 1921; Hawkins and Schild, 1951; de Candole *et al.*, 1953) and diminish the rate of flow in perfused bronchial tree preparations (Swanson and Webster, 1930; Cotui, Burstein and Wright, 1936). Sollmann and Gilbert (1937) made microscopic observations on thin sections of lung and found that application of eserine caused bronchiolar contraction.

Comparison of the changes in tidal air volume occurring in the present experiments and in those on the entire animal reported by M. de B. Daly (1957) shows that in the perfused lung preparations the responses were slower in onset and were on the whole smaller in size even though the concentrations of the injected drugs were often higher, assuming even distribution throughout the animal. There are several plausible explanations for this. It may be that, in the entire animal, the concentration of acetylcholine in the lung after poisoning is higher because of the amount formed locally at vagus nerve endings by the tonic activity of the vagus on the bronchioles (Roy and Brown, 1885; Chauveau, 1889; M. de B. Daly and Schweitzer, 1952) and also because considerable quantities of acetylcholine may be conveyed to the lungs from other sites such as the bowel (Douglas and Paton, 1954). Furthermore, there is the possibility suggested by M. de B. Daly (1957) that an increase in vagal bronchomotor tone may occur in anticholinesterase poisoning as a result of a central action of asphyxia consequent upon depression of pulmonary ventilation. Such a mechanism cannot operate in the isolated perfused lung preparation.

In the entire animal ventilated artificially at constant peak inflationary pressure atropine

occasionally did not completely restore the tidal air volume to its original value after poisoning with an anticholinesterase (Holmstedt, 1951; M. de B. Daly, 1957; M. de B. Daly and Wright, 1957). In the present experiments this well-known antagonistic effect of atropine was confirmed, but again there were several instances in which the tidal air volume failed to return to normal; the compliance of the lungs remained reduced although the expiratory resistance returned to normal, suggesting that parts of the lung were not now being ventilated. Whether this was due to failure of some of the obstructed bronchioles to relax or to some other effect preventing the return of lung movements is not altogether clear. The bronchial circulation which supplies arterial blood to the bronchial tree was not perfused in these experiments, so that pulmonary arterial blood would supply not only tissues in and beyond the respiratory bronchioles but probably some of the bronchi and bronchioles as well (Ghoreyeb and Karsner, 1913). Pulmonary artery blood at relatively low pressure would reach these structures by back-flow through communicating vessels between the bronchial and pulmonary vascular systems situated in the region of the respiratory bronchioles (Küttner, 1878; Miller, 1925). Since the blood vessels supplying the bronchial tree pass freely through the bronchial musculature, constriction of the bronchial muscles by anticholinesterases might conceivably cut off their blood supply and so prevent access to them of drugs subsequently injected. An alternative explanation is that contraction of the bronchioles and increased glandular secretion produced by anticholinesterases renders complete closure of airways more likely, with the result that, when atropine is given and the bronchial muscles relax, these airways fail to open again owing to the effect of surface tension. Much greater forces would then be necessary to reopen these airways (Radford and Lefcoe, 1955). In our preparations in which atropine only partially restored respiratory movements, it was found that inflation of the lungs once or twice at increased pressure caused the compliance to return to normal, or very nearly so, on establishing the original respiratory pressure.

Pulmonary Vascular Effects.—Our results have shown that both sarin and TEPP cause an increase in pulmonary vascular resistance. Alcock *et al.* (1935) observed a similar effect on injection of eserine in isolated perfused dog lungs, and Bhattacharya and Pochet (1956) have reported recently that sarin caused a slowing of perfusion through the isolated lung of the guinea-pig.

In the present experiments the increase in pulmonary vascular resistance was nearly always accompanied by bronchoconstriction, and this raises the question as to how far the vascular response is the result of a direct action of the drugs on the pulmonary blood vessels or of a passive effect of a change in intrapulmonary pressure. Our findings would suggest that the observed vascular and bronchomotor effects are, at least in part, independent of each other, because in one experiment (No. 7 of Table I) an increase in pulmonary arterial pressure occurred in response to injection of sarin with no alteration in tidal air volume. Examination of the records of other experiments showed that there was no consistent relationship between the changes in tidal air volume and in pulmonary arterial pressure or left atrial outflow. Furthermore, similar changes in vascular resistance were obtained in unventilated lungs. Our experiments, however, provide no evidence that would enable us to state which parts of the pulmonary vascular bed are responsible for these effects.

A systematic study of the effects of acetylcholine on the pulmonary vascular bed has not been made. From the literature, small doses of acetylcholine in the dog usually produce pulmonary vasodilatation and large doses vasoconstriction, either response being enhanced by eserine and abolished by atropine (I. de B. Daly and Euler, 1932; Gaddum and Holtz, 1933; Alcock *et al.*, 1935). A reduction in pulmonary outflow in response to large doses of acetylcholine was also found by Tronci (1934). Franklin (1932) showed, in isolated rings of blood vessels taken from the dog, that acetylcholine caused relaxation of extrapulmonary arteries but contraction of the veins. Responses of intrapulmonary vessels were variable, but contraction predominated in both types. In a small number of experiments reported here the smallest effective dose of acetylcholine always produced pulmonary vasoconstriction which was enhanced by both sarin and TEPP.

Our experiments suggest that the site of action of sarin and TEPP is peripheral to the ganglia because these agents caused bronchoconstriction and an increase in pulmonary vascular resistance in preparations pretreated with hexamethonium. This peripheral effect could be the result of a direct action of the anticholinesterases or to accumulation of endogenous acetylcholine. In this connexion, the earlier work of Thornton (1934) and of Kordik, Bülbring and Burn (1952) suggested a slow synthesis of acetylcholine is present in the lung. Thornton (1934) found on

stimulation of the cervical vagus nerve a substance in the effluent from the perfused guinea-pig lung with the properties of acetylcholine, and Kordik *et al.* (1952) presented evidence that acetylcholine was synthesized by non-nervous elements in the mucous membrane of the rabbit trachea. The possibility that bronchoconstriction occurring in response to anticholinesterases is in part the result of an action on intrapulmonary vagal ganglia (Heymans, 1949) is not ruled out by our experiments.

The bronchoconstrictor and pulmonary vasoconstrictor responses occurring on injection of anticholinesterases are antagonized by atropine. In a few experiments the efficacy of adrenaline, noradrenaline and isoprenaline in reversing the bronchoconstriction produced by sarin and TEPP was tested. These drugs were found to have smaller and more evanescent effects than atropine. Furthermore, whereas atropine and isoprenaline caused pulmonary vasodilatation after poisoning, the injection of adrenaline and noradrenaline, in confirmation of the findings of other workers (see I. de B. Daly, 1933; also Konzett and Hebb, 1949), led to a rise in pulmonary arterial pressure and a diminution in lung blood volume. It must be pointed out, however, that an assessment of the value of a drug antagonizing the pulmonary circulatory effects of anticholinesterases cannot be made solely from observations obtained in the isolated organ, but must take into consideration passive cardiomotor and respiratory effects influencing the vascular bed. These points are considered more fully elsewhere (M. de B. Daly, 1957).

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References

Alcock, P., Berry, J. L., and Daly, I. de Burgh (1935). *Quart. J. exp. Physiol.*, **25**, 369.

Bayliss, L. E., and Robertson, G. W. (1939). Ibid., **29**, 27.

Bhattacharya, B. K., and Pochet, A. (1956). *Arch. int. Pharmacodyn.*, **107**, 473.

Chauveau (1889). *Sem. Médicale*, p. 117.

Comroe, J. H., Jr., Nisell, O. I., and Nims, R. G. (1954). *J. appl. Physiol.*, **7**, 225.

Cotui, Burstein, C. L., and Wright, A. M. (1936). *J. Pharmacol.*, **58**, 33.

Daly, I. de Burgh (1928). *J. Physiol.*, **65**, 422.

—— (1933). *Physiol. Rev.*, **13**, 149.

—— and Euler, U. S. von (1932). *Proc. roy. Soc., B*, **110**, 92.

Daly, M. de Burgh (1957). *J. Physiol.*, **139**, 250.

—— and Schweitzer, A. (1952). Ibid., **116**, 35.

—— and Wright, P. G. (1957). Ibid., **139**, 273.

de Candole, C. A., Douglas, W. W., Evans, C. L., Holmes, R., Spencer, K. E. V., Torrance, R. W., and Wilson, K. M. (1953). *Brit. J. Pharmacol.*, **8**, 466.

Dixon, W. E., and Brodie, T. G. (1903). *J. Physiol.*, **29**, 97.

Douglas, W. W., and Paton, W. D. M. (1954). Ibid., **124**, 325.

Franklin, K. J. (1932). Ibid., **75**, 471.

Gaddum, J. H. (1939). Ibid., **67**, 16P.

—— and Holtz, P. (1933). Ibid., **77**, 139.

Ghoreyeb, A. A., and Karsner, H. T. (1913). *J. exp. Med.*, **18**, 500.

Green, R. E., McKay, E. A., and Krop, S. (1947). *Fed. Proc.*, **6**, 334.

Hawkins, D. F., and Schild, H. O. (1951). *Brit. J. Pharmacol.*, **6**, 682.

Hebb, C. O., and Konzett, H. (1949). *J. Pharmacol.*, **96**, 228.

Heymans, C. (1949). *Bull. Acad. Med. Belg.*, **14**, 76.

Holmstedt, B. (1951). *Acta physiol. scand.*, **25**, Suppl. 90.

Konzett, H., and Hebb, C. O. (1949). *Arch. int. Pharmacodyn.*, **78**, 210.

—— and Rössler, R. (1940). *Arch. exp. Path. Pharmak.*, **195**, 71.

Kordik, P., Bülbring, E., and Burn, J. H. (1952). *Brit. J. Pharmacol.*, **7**, 67.

Küttner, C. (1878). *Virchows Arch.*, **73**, 476.

Macht, D. I., and Ting, G. C. (1921). *J. Pharmacol.*, **18**, 373.

Miller, W. S. (1925). *Ann. Rev. Tuberc.*, **12**, 87.

Mount, L. E. (1956). *J. Physiol.*, **131**, 393.

Radford, E. P., Jr., and Lefcoe, N. M. (1955). *Amer. J. Physiol.*, **180**, 479.

Roy, C. S., and Brown, G. (1885). *J. Physiol.*, **6**, 21P.

Sollmann, T., and Gilbert, A. J. (1937). *J. Pharmacol.*, **61**, 272.

Swanson, E. E., and Webster, R. K. (1930). Ibid., **38**, 327.

Thornton, J. W. (1934). *J. Physiol.*, **82**, 14P.

Tronci, L. (1934). *Sperimentale*, **88**, 719.

Brit. J. Pharmacol. (1957), **12**, 513.

THE OXIDATION OF CYSTAMINE AND HOMOCYSTAMINE BY MAMMALIAN ENZYMES

BY

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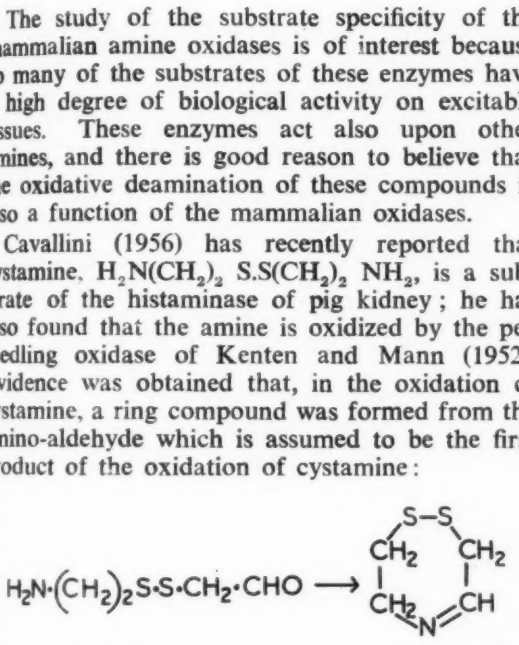
The oxidative deamination of cystamine and homocystamine by mammalian oxidases has been studied. The histaminase of pig kidney oxidizes homocystamine much more slowly than cystamine. The amine oxidase of mammalian liver (guinea-pig, rabbit) oxidizes homocystamine more rapidly than cystamine. Both amines are oxidized by plasma (or serum) of ruminants (ox, sheep, goat) and of the horse. In the enzymatic oxidation of homocystamine both amino-groups are removed; there is no evidence that a ring compound analogous to cystaldimine is accumulating.

The study of the substrate specificity of the mammalian amine oxidases is of interest because so many of the substrates of these enzymes have a high degree of biological activity on excitable tissues. These enzymes act also upon other amines, and there is good reason to believe that the oxidative deamination of these compounds is also a function of the mammalian oxidases.

Cavallini (1956) has recently reported that cystamine, $H_2N(CH_2)_2\ S.S(CH_2)_2\ NH_2$, is a substrate of the histaminase of pig kidney; he has also found that the amine is oxidized by the pea seedling oxidase of Kenten and Mann (1952). Evidence was obtained that, in the oxidation of cystamine, a ring compound was formed from the amino-aldehyde which is assumed to be the first product of the oxidation of cystamine:



This seven-membered ring compound was called cystaldimine (Cavallini, De Marco, and Mondovì, 1956, 1957).

Cystamine is of interest because cysteamine, its reduced form, is a constituent of co-enzyme A, and because it has a protective action against radiations (Bacq, Herve, Lecomte, Fischer, Blavier, Dechamps, Le Bihan, and Rayet, 1951). We have, therefore, studied the oxidation of cystamine by a number of mammalian oxidases. These oxidases included the amine oxidase of mammalian liver and also two other enzymes which have a substrate specificity similar to that of liver oxidase. These are the spermine oxidase of ruminant plasma (Hirsch, 1953; Tabor, Tabor and Rosenthal, 1954) and an enzyme recently found in horse serum and provisionally called benzylamine oxidase (Bergeret, Blaschko, and Hawes, 1957). Both spermine oxidase and benzylamine oxidase also act slowly on 5-hydroxytryptamine and on sympathomimetic amines.

Through the kindness of Dr. R. Cecil, we have recently obtained a small amount of homocystamine dihydrobromide, $H_2N(CH_2)_3\ S.S(CH_2)_3\ NH_2 \cdot 2\ HBr$, and we have studied the oxidation of this compound by the mammalian oxidases, in comparison with that of cystamine.

METHODS

The preparations of histaminase (an extract of an acetone-dried powder of pig kidney) and of liver amine oxidase did not differ from those already described (Barlow, Blaschko, Himms, and Trendelenburg, 1955). Horse serum and ruminant plasma (or serum) were dialysed against 0.067 M sodium phosphate buffer; usually the *p*H of the buffer was 7.4, but in a few experiments it was 6.5.

Uptake of oxygen was measured manometrically at a temperature of 37.5° and in an atmosphere of oxygen. In those experiments in which the initial uptake was measured, substrate concentration was 10^{-2} M; but when the reaction was followed to completion, substrate concentrations were lower.

Ammonia was determined using the microdiffusion method, followed by colorimetric determination with phenate-hypochlorite (Conway, 1947).

Results

The observations of Cavallini *et al.* (1956) on the oxidation of cystamine by pig kidney extract were readily confirmed. The extracts oxidized homocystamine at a very much slower rate. In one of these experiments (at *p*H 7.4), the oxygen uptake of 1 ml. of extract incubated at 37.5° for 15 min. without added substrate was 8.5 μl. O_2. The additional amounts of oxygen consumed in the presence of 10^{-2} M cadaverine, cystamine, and homocystamine were 31.5, 37, and 4 μl. O_2 respectively.

Rabbit and guinea-pig liver preparations slowly oxidized both cystamine and homocystamine ; in every experiment the rate of oxygen uptake with homocystamine exceeded that with cystamine. Thus in an experiment with guinea-pig liver enzyme the amounts of oxygen consumed in the first 15 min. in the presence of 10^{-2} M tyramine, cystamine, and homocystamine were 156.5, 14.5, and 21 μl. O_2 respectively ; in a similar experiment with rabbit liver the amounts consumed were 106, 7.5, and 14.5 μl. O_2.

No significant uptake of oxygen occurred when human plasma was incubated with either cystamine or homocystamine. However, the amines were oxidized by horse serum. There is no spermine oxidase in horse serum, but we have already referred to the observation that it contains another oxidase which acts on many amines such as 5-hydroxytryptamine and benzylamine

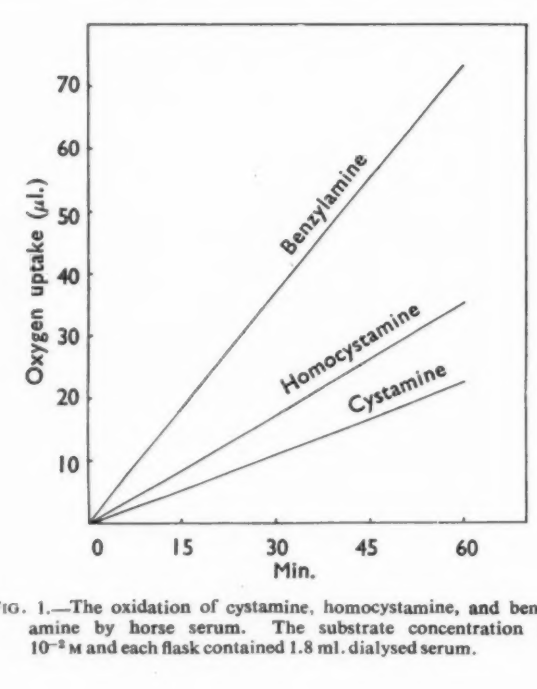


FIG. 1.—The oxidation of cystamine, homocystamine, and benzylamine by horse serum. The substrate concentration was 10^{-2} M and each flask contained 1.8 ml. dialysed serum.

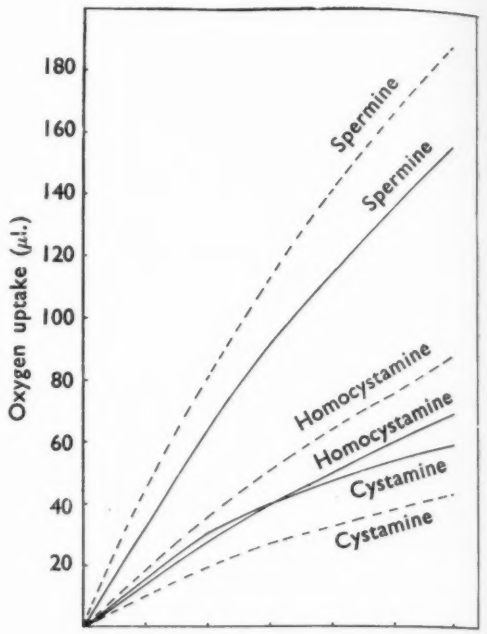


FIG. 2.—The oxidation of cystamine, homocystamine, and spermine by bovine serum. One sample of serum was dialysed against phosphate buffer of *p*H 7.4 (solid lines); another sample of the same serum against phosphate buffer of *p*H 6.5 (broken lines). The substrate concentration was 10^{-2} M. Each flask contained 1.0 ml. dialysed serum.

that are also oxidized by the amine oxidase of liver and other mammalian tissues (Bergeret *et al.*, 1957). Fig. 1 shows that homocystamine was oxidized more rapidly than cystamine.

With bovine serum, there was a difference in the rates of oxygen uptake at different *p*H. At *p*H 7.4, homocystamine and cystamine were oxidized at similar rates, but at *p*H 6.5 homocystamine was the more rapidly oxidized (Fig. 2). Sheep serum, which is known to contain spermine oxidase (Hirsch, 1953), also acted upon both cystamine and homocystamine. We have found that spermine oxidase is also present in goat plasma which oxidizes homocystamine more rapidly than cystamine (*p*H 7.4).

In all these experiments, a brown colour appeared in the flasks that were incubated with cystamine ; this is similar to the observation of Cavallini *et al.* (1957) with pig kidney extract. No colour appeared with homocystamine as substrate.

In their experiments with pig kidney extract, Cavallini *et al.* (1957) found that the reaction came to a standstill when one molecule of ammonia had been formed for every molecule of cystamine added. They found that in the

TABLE I

OXYGEN CONSUMPTION AND AMMONIA FORMATION IN THE OXIDATIVE DEAMINATION OF CYSTAMINE AND HOMOCYSTAMINE

Substrate concentration, $1{\cdot}25\times10^{-3}$ M; *o*-aminobenzaldehyde (*o*ABA) concentration, $1{\cdot}67\times10^{-3}$ M. The values are expressed in moles of O_2 consumed and NH_3 formed/mole of added substrate. * Indicates estimations at *p*H 6·5.

Substrate	Cystamine				Homocystamine			
	Oxygen Uptake		Ammonia Formation		Oxygen Uptake		Ammonia Formation	
Source of Enzyme	Without *o*ABA	With *o*ABA	Without *o*ABA	With *o*ABA	Without *o*ABA	With *o*ABA	Without *o*ABA	With *o*ABA
Pig kidney ..	0·68	0·54	1·37	0·83	—	—	—	—
Ox serum ..	1·10	—	—	—	1·98*	—	—	—
" " ..	0·93	—	1·60	—	1·71*	—	1·94*	—
" " ..	1·11	0·57	1·72	0·89	—	—	—	—
" " ..	1·44	0·68	1·53	0·76	1·40	1·56	1·91	1·74
" " ..	—	—	—	—	1·95	1·82	1·74	2·00
Goat plasma ..	1·02	0·80	1·74	1·40	1·84	1·81	1·80	1·97

presence of *o*-aminobenzaldehyde a yellow pigment was formed; this was analogous to the observations of Tabor (1951) on putrescine and cadaverine.

In Table I we have summarized our observations on the relation of oxygen uptake and ammonia formation in the absence and in the presence of *o*-aminobenzaldehyde. In these experiments, the manometer flasks contained 2 ml. of fluid, the concentration of amine (cystamine or homocystamine) was 1.25×10^{-3} M; the *o*-aminobenzaldehyde concentration was 1.67×10^{-3} M.

It can be seen that the oxygen uptake with ox serum and with cystamine as substrate was between 0.93 and 1.44 moles of O_2/mole of substrate; ammonia formation was between 1.53 and 1.72/mole of substrate. In other words, there was evidence for the removal of more than one of the two amino groups. In the presence of *o*-aminobenzaldehyde, both oxygen uptake and ammonia formation were reduced: O_2 uptake was 0.57 and 0.68 moles in two experiments; ammonia formation was 0.89 and 0.76 moles.

These results are consistent with the interpretation that, in the presence of *o*-aminobenzaldehyde, cystaldimine was accumulating. In the absence of the reagent, where ammonia formation was near two moles/mole of cystamine, oxygen consumption was close to one mole. This suggested that an aldehyde was accumulating as an end-product of the oxidation. In two experiments with ox serum as source of enzyme and cystamine as substrate, at the end of the incubation and after deproteinization of the incubation mixture with trichloroacetic acid, we added a saturated solution of 2:4-dinitrophenylhydrazine in 2 N HCl. A slight colour change to orange occurred and a very slight precipitate formed, indicating that some carbonyl compound was accumulating in the mixture.

Table I also gives our results with homocystamine. There was no evidence of cyclization. In the presence as well as in the absence of *o*-aminobenzaldehyde, ammonia formation and oxygen consumption were higher than with cystamine as substrate. Ammonia formation was between 1.74 and 2.00 moles/mole of homocystamine, indicating that the second amino-group was also attacked. In the experiments with homocystamine, there was no evidence of the formation of a hydrazone when 2:4-dinitrophenylhydrazine was added after the incubation.

In some of the experiments with cystamine, oxygen consumption had not entirely stopped at the end of incubation.

In the presence of *o*-aminobenzaldehyde, a yellow colour developed in the flasks incubated with cystamine as well as in those incubated with homocystamine.

DISCUSSION

The experiments described show that both cystamine and homocystamine are oxidized by mammalian enzymes. The oxidation of cystamine by the histaminase of pig liver, described by Cavallini (1956), was confirmed. In addition, we have found that cystamine is a substrate of two other oxidases, the spermine oxidase of ruminant plasma and the benzylamine oxidase of horse serum. In most species examined, the plasma enzymes acted more rapidly on homocystamine than on cystamine. The oxidation of cystamine by preparations of mammalian liver was slow; homocystamine was attacked at a slightly faster rate.

Our knowledge of the metabolism of cystamine has recently been reviewed (Eldjarn and Pihl, 1956; Cavallini, 1956). It is not certain whether or not cystaldimine can appear as an end-product of metabolism, but in our experiments the total

formation of ammonia, in the absence of *o*-aminobenzaldehyde, always exceeded one mole for each mole of cystamine added. It is likely that a considerable amount of amine was oxidized beyond the cystaldimine stage.

With homocystamine as substrate, a yellow colour appeared in the presence of *o*-aminobenzaldehyde, but otherwise there was no indication that a ring compound was accumulating.

Homocystamine was less rapidly oxidized than cystamine by the pig kidney enzyme. This is in agreement with what is known of the action of histaminase on polymethylene diamines. The slightly higher rate of oxidation of homocystamine by mammalian liver can also be interpreted on the basis of similar observations with the polymethylene diamines (Blaschko and Hawkins, 1950). The two plasma enzymes, spermine oxidase and benzylamine oxidase, acted upon both cystamine and homocystamine. Their specificity requirements are not so well known, but it appears that their position is intermediate between that of liver amine oxidase and histaminase.

To what extent histaminase takes part in the breakdown of cystamine is not yet known ; the present experiments show that for some mammalian species the action of the plasma oxidases must also be considered.

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References

Bacq, Z. M., Herve, A., Lecomte, J., Fischer, P., Blavier, J., Dechamps, G., Le Bihan, H., and Rayet, P. (1951). *Arch. int. Physiol.*, **59**, 442.

Barlow, R. B., Blaschko, H., Himms, J. M., and Trendelenburg, U. (1955). *Brit. J. Pharmacol.*, **10**, 116.

Bergeret, B., Blaschko, H., and Hawes, R. (1957). *Nature, Lond.*, in the press.

Blaschko, H., and Hawkins, J. (1950). *Brit. J. Pharmacol.*, **5**, 625.

Cavallini, D. (1956). *Colloque sur la Biochimie du Soufre*, p. 208. Paris: Editions du Centre National de la Recherche Scientifique.

—— De Marco, C., and Mondovì, B. (1956). *Experientia*, **12**, 377.

—— —— —— (1957). *Biochim. Biophys. Acta*, **24**, 353.

Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, p. 96. London: Crosby Lockwood & Son.

Eldjarn, L., and Pihl, A. (1956). *Colloque sur la Biochimie du Soufre*, p. 217. Paris: Editions du Centre National de la Recherche Scientifique.

Hirsch, J. G. (1953). *J. exp. Med.*, **97**, 345.

Kenten, R. H., and Mann, P. J. G. (1952). *Biochem. J.*, **50**, 360.

Tabor, C. W., Tabor, H., and Rosenthal, S. M. (1954). *J. biol. Chem.*, **208**, 645.

Tabor, H. (1951). Ibid., **188**, 125.

Brit. J. Pharmacol. (1957), **12**, 517.

METABOLIC AND OTHER EFFECTS OF RESERPINE UPON SMOOTH AND SKELETAL MUSCLE

BY

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Reserpine increased the concentration of potassium ions in the fluid bathing the isolated frog sartorius muscle. The effect of reserpine upon respiration and upon glycolysis in rabbit intestinal muscle has been investigated using the Warburg "Direct" method. After a latent period, reserpine (50 μg./ml.) depressed respiration but did not affect glycolysis in gut muscle. These results, together with previous observations, may point to an effect of reserpine upon carbohydrate metabolism.

Earlier work (Gillis and Lewis, 1956a, 1956b, 1957) indicated that reserpine affected metabolic processes which supplied energy for the contraction of smooth muscle. It was shown by Gillis and Lewis (1956b, 1957) that certain intermediates of carbohydrate metabolism antagonized the depression by reserpine of acetylcholine- and histamine-induced contractions of guinea-pig ileum. Intermediates of fat and protein metabolism were ineffective. If reserpine had a metabolic site of action it seemed reasonable to investigate whether this was reflected in a decreased ability of the muscle to utilize glucose. The potassium ion (K^+) is known to participate in the activation of certain important enzyme systems involved in carbohydrate metabolism (Sheppard, 1951). Changes in the extra- and intra-cellular concentrations of K^+ might be observed if reserpine interfered with the intermediary metabolism of carbohydrate. It is well established that contractions of smooth (Born and Bülbring, 1956), cardiac (O'Brien and Wilde, 1952) and skeletal (Fenn, 1940; Hahn and Hevesy, 1941; Noonan, Fenn, and Haege, 1941) muscle are associated with an increase in the K^+ concentration of the fluid bathing the tissue. This appears to be due to an increased efflux of potassium ions rather than to a decreased influx, since adrenaline-induced relaxation in intestinal smooth muscle is accompanied by an increased inward movement of K^+ (Born and Bülbring, 1956). Reserpine causes a contraction of the isolated frog rectus abdominis muscle (Gillis and Lewis, 1956a; Barrett, Baker, and Plummer, 1956) and of the isolated rabbit duodenum (Gillis and Lewis, 1957). The time-characteristics of the contraction of the frog rectus muscle led us to consider the possibility that reserpine was causing an increase in the concentration of K^+ in the extracellular fluid.

METHODS

Preparation of Solutions.—For experiments on the frog sartorius muscle, reserpine was dissolved in an aqueous solution of 10% ascorbic acid to give a concentration of 2 mg./ml. The *p*H was about 2.5, but was adjusted, by the addition of small volumes of 5% $NaHCO_3$ solution, to 4.5 immediately before use. The mixture was diluted with frog Ringer solution to give a final concentration of reserpine of 1 mg./ml. The control solution was prepared by treatment of 10% ascorbic acid solution in the same way.

For the determination of respiration and glycolysis, a 5 mg./ml. solution of reserpine was prepared in 5% aqueous tartaric acid. The *p*H of a 2 ml. aliquot was adjusted to 4.5 with $NaHCO_3$ and the mixture diluted to 7.4 ml. Addition of 0.1 ml. of this solution from the side arm gave a final flask concentration of 10 μg./ml. reserpine.

Isolated Frog Sartorius Muscle.—The radioactive material was obtained as a 1.15% solution of ^{42}KCl. Frogs received 1 ml. of this solution by injection into the dorsal lymph sac.

After a 2 hr. equilibration period the frogs were decapitated and pithed, the skin over the thigh removed and the rectus abdominis muscle carefully freed at the point of insertion into the pelvic girdle. The upper tendinous attachment of the sartorius muscle was thus exposed and the proximal and distal attachments then freed from underlying tissues. Cotton threads were tied around both ends of the sartorius muscle, which was freed from the femur and pelvic girdle and finally from the underlying muscles. Two types of experiment were performed. In the

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first, the muscle was suspended in a test-tube containing 10 ml. frog Ringer solution (NaCl, 0.65%; KCl, 0.014%; $CaCl_2$, 0.012%; $NaHCO_3$, 0.02%; dextrose, 0.2%) at room temperature and through which O_2 was bubbled. Two parallel series of nine tubes were set up. One series contained 50 μg./ml. reserpine in frog Ringer solution; the other contained an equivalent volume of the control solution. One muscle from each pair was used in the reserpine series, the other in the control series. The muscles were suspended by a thread from a wire support at the top of the tube. They were stretched with a 2 g. weight tied to the threads at their lower ends. At 20 min. intervals each muscle was transferred to the next tube in the series. The ^{42}K in the bathing fluid was measured with a Geiger-Müller liquid counter (type M.6). The results in counts/min. were corrected for background and lost counts. Allowance was made for decay and the final value for the activity converted to parts/million of ^{42}K. In the second type of experiment, the muscle was suspended in a 50 ml. bath containing frog Ringer solution. The bath fluid was circulated continuously by a pump, through an F.M.6 flow counter, cooled, and thence returned to the bath. A continuous integrated record of the ^{42}K in the bathing fluid was obtained with a "Labgear" recording count ratemeter. An increase in ^{42}K in the bathing fluid was recorded as an increased gradient of the tracing. Muscle tone was recorded simultaneously by conventional methods on a smoked surface.

Respiration and Glycolysis in Isolated Rabbit Intestine.—Rabbits weighing between 1.5 kg. and 2.1 kg. and between 4 and 9 months old were used. About 60 cm. of small intestine was rapidly removed. For experiments on respiration, it was moistened with ice-cold modified Krebs-Hensleit solution (NaCl, 0.692%; KCl, 0.035%; $CaCl_2$, 0.029%; KH_2PO_4, 0.016%; $MgSO_4.7H_2O$, 0.029%), or, for experiments on glycolysis, with Krebs-bicarbonate solution (NaCl, 0.692%; KCl, 0.035%; $CaCl_2$, 0.029%; KH_2PO_4, 0.016%; $NaHCO_3$, 0.21%; $MgSO_4.7H_2O$, 0.029%; dextrose, 0.2%). The segment of intestine was opened along the line of attachment of the mesentery, stretched gently and, with the mucosa upwards, pinned at its four corners upon a dissecting board. The mucosa was removed as completely as possible by scraping gently with a scalpel blade. Two incisions were made about 2 mm. from and parallel to both outer edges of the strip. The tissue remaining between the incisions was cut into pieces, each weighing about 100 mg. These were used in the experiments to be described. Experiments on oxygen consumption and on glycolysis were carried out by means of the Warburg "Direct" method, using either an atmosphere of O_2 (respiration) or 95% N_2 and 5% CO_2 (glycolysis). *For respiration,* the main chamber of each Warburg flask contained 2.2 ml. modified Krebs-Hensleit solution. The centre well contained 0.2 ml. 20% KOH. Since the addition of saline/reserpine or saline/control mixtures from the side arm would cause a slight change of the final salt concentration in the main compartment, the composition of the physiological saline in the side arms was suitably adjusted to avoid any change. *For glycolysis,* the main chamber of the flask contained 2.4 ml. of Krebs-bicarbonate solution. Similar precautions to those outlined above were taken to avoid alteration in the final ionic concentration. Reserpine was added from the side arm to give final concentrations of 10 or 50 μg./ml. In control experiments, an equivalent volume of tartaric acid control solution was added from the side arm. After tipping, the total volume of the reaction mixture together with the piece of tissue was 3 ml.

Results

Frog Sartorius Muscle.—In all experiments reserpine, in doses of 20 and 50 μg./ml., caused a marked increase in the ^{42}K content of the bath fluid ($^{42}K_b$) (Fig. 1, Table I). A slow contraction of the muscle accompanied this phenomenon (Fig. 2). Less than 20 μg./ml. reserpine did not consistently produce a contraction of the muscle.

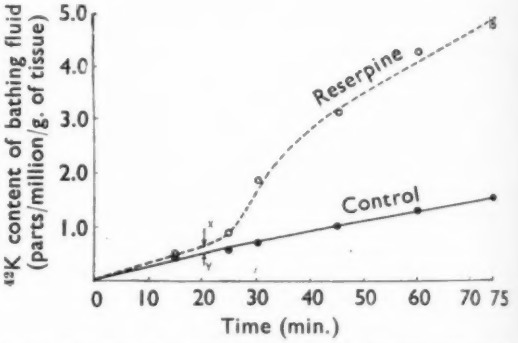

FIG. 1.—The effect of reserpine upon the ^{42}K concentration of the fluid bathing an isolated frog sartorius muscle. At X, reserpine (50 μg./ml.) was added. At Y, the control solution was added.

TABLE I

^{42}K CONTENT OF FLUID BATHING THE FROG SARTORIUS MUSCLE WHEN RESERPINE OR THE CONTROL SOLUTION WAS ADDED

All values (±S.D.) are in parts/million and were calculated from tracings obtained with a "Labgear" recording count ratemeter.

	Time after Addition of Reserpine or Control Solution (min.)					
	15	25	30	45	60	75
Reserpine 50 μg./ml. (mean of 9 experiments) ..	0·49 (±0·25)	0·90 (±0·39)	1·81 (±1·09)	3·14 (±1·69)	4·28 (±1·92)	4·88 (±1·96)
Control (mean of 10 experiments)	0·41 (±0·17)	0·59 (±0·30)	0·70 (±0·36)	1·00 (±0·48)	1·29 (±0·53)	1·55 (±0·71)

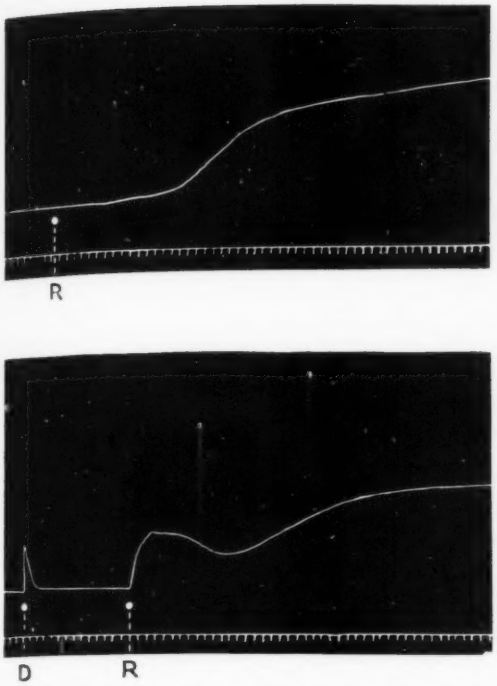

FIG. 2.—The stimulant action of reserpine upon the isolated frog sartorius muscle. Upper record, reserpine 50 μg./ml. alone at R; lower record, reserpine 50 μg./ml. at R after 20 μg./ml. decamethonium at D. The biphasic response to reserpine shown in the lower record was not always seen. Time, 30 sec.

Both the appearance of the contraction and the increased $^{42}K_b$ were preceded by a latent period of 4 to 11 min. The contraction was maintained only so long as reserpine remained in contact with the muscle. (+)-Tubocurarine (25 μg./ml.) increased the latent period by as much as 100%. Decamethonium (20 μg./ml.) reduced or abolished it (Fig. 2). Neither the magnitude of the contraction nor the increased $^{42}K_b$ was altered by tubocurarine or decamethonium. Neither drug had any effect upon an established reserpine contraction. 2:4-Dinitrophenol (0.2 mM), a concentration shown by Maizels (1951) to cause a release of potassium from frog skeletal muscle, was ineffective when ordinary frog Ringer solution was used, but caused an increase in the $^{42}K_b$ when a potassium-free solution was substituted. Thus there appears to be some difference in the mechanism of potassium release between reserpine and 2:4-dinitrophenol.

When test and control muscles were suspended in parallel series of nine tubes (see Method), a marked increase in $^{42}K_b$ was seen in the test series. The release of potassium by the ascorbic acid solution in the control series was not greater than that seen in experiments in which the muscles were suspended in frog Ringer solution. The increase in $^{42}K_b$ was seen only when the muscle was immersed in a solution containing reserpine: it fell to control values on transferring to the reserpine-free solution.

Warburg Experiments.—The addition of 0.2% dextrose did not influence the $Q_{O_2}^{O_2}$ values over the first, second, or third hours of the experiment. This presumably indicated that the tissue had sufficient endogenous glucose to support aerobic metabolism. Dextrose was therefore omitted from the physiological salines in these experiments.

Reserpine (50 μg./ml.) reduced significantly ($P=0.05$) the oxygen uptake of pieces of rabbit intestinal muscle when compared with the control values for tissue incubated in modified Krebs-Hensleit solution. There was a latent period of 20 min. before a difference was observed between flasks containing reserpine and those containing the control (Fig. 3, Table II).

Reserpine (10 μg./ml.) and the control solution of tartaric acid, equivalent to 10 μg. and 50 μg./ml. of reserpine, had no demonstrable effect upon respiration.

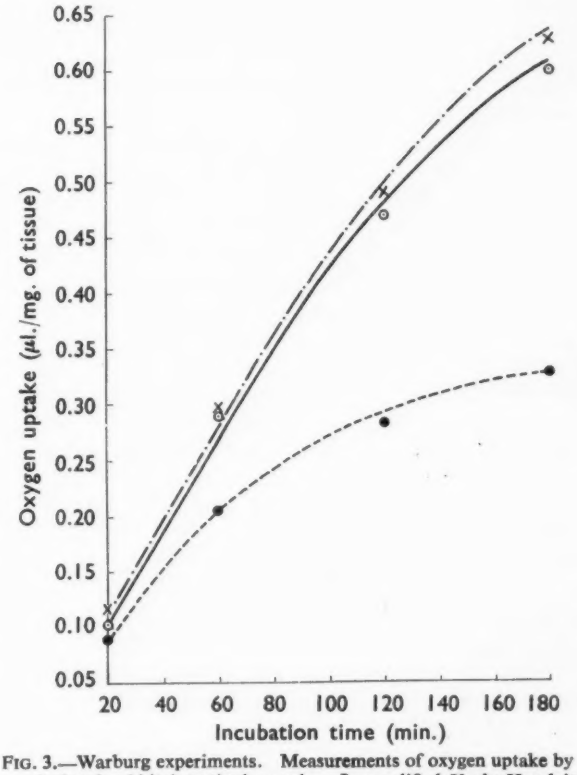

FIG. 3.—Warburg experiments. Measurements of oxygen uptake by isolated rabbit intestinal muscle. In modified Krebs-Hensleit control solution X–·–X; in tartaric acid control solution (equivalent to 50 μg./ml. of reserpine) ⊙———⊙; and in reserpine (concentration of 50 μg./ml.) ●– – –●.

TABLE II

THE EFFECT OF RESERPINE UPON RESPIRATION IN ISOLATED RABBIT INTESTINAL MUSCLE

The oxygen uptake in μl. ($\pm$S.D.)/mg. of tissue at each time is the average of eight experiments.

	Incubation Time (Min.)			
	20	60	120	180
Modified Krebs-Hensleit solution ..	0·116 (±0·062)	0·296 (±0·114)	0·491 (±0·145)	0·629 (±0·219)
Reserpine 50 μg./ml.	0·090 (±0·021)	0·206 (±0·086)	0·283 (±0·129)	0·330 (±0·143)
,, 10 ,, ...	0·114 (±0·055)	0·264 (±0·140)	0·460 (±0·278)	0·550 (±0·291)
Tartaric acid solution equivalent to				
(a) 50 μg./ml. reserpine	0·105 (±0·004)	0·292 (±0·108)	0·469 (±0·158)	0·600 (±0·231)
(b) 10 ,, ,, ...	0·097 (±0·045)	0·306 (±0·120)	0·511 (±0·217)	0·618 (±0·218)

Reserpine (10 and 50 μg./ml.) had no significant effect ($P=0.05$) upon the glycolytic activities of isolated rabbit intestinal muscle.

DISCUSSION

Reserpine has been shown to depress both spontaneous and drug-induced activity in isolated skeletal, visceral, and cardiac muscle (Gillis and Lewis, 1956a, 1956b, 1957). Previous evidence points to a relationship between reserpine and carbohydrate metabolism in the smooth muscle of guinea-pig ileum (Gillis and Lewis, 1956b, 1957). Under suitable conditions of bath *p*H, certain intermediates of carbohydrate metabolism antagonized the depression by reserpine of drug-induced contractions, whilst intermediates of fat and protein metabolism were inactive.

Anoxia has been shown to cause either a reduction in, or complete inhibition of, spontaneous tone and rhythmic activity in isolated rabbit intestinal muscle (West, Hadden, and Farah, 1951), and in the taenia coli muscle of the guinea-pig (Born, 1956). At the same time the ability of the tissue to maintain electrically-induced (Born, 1956) or drug-induced (West *et al.*, 1951) tone was completely abolished.

The effects of reserpine upon spontaneous and drug-induced tone are similar in many respects to those of experimentally produced anoxia. It is possible that reserpine in effect renders the intestinal smooth muscle of the guinea-pig ileum anoxic by virtue of its ability to interfere with the energy-producing reactions of carbohydrate metabolism.

An effect upon the oxidative metabolism of carbohydrate is supported by our observations that reserpine, after a latent period, decreased oxygen-uptake in isolated rabbit intestinal muscle and caused an increased release of potassium from frog skeletal muscle.

Krebs, Eggleston and Terner (1951) have shown that guinea-pig brain and kidney cortex retain potassium only in the presence of (−)-glutamic acid and α-ketoglutaric acid (to which the former can easily be converted). The presence of a substance which interferes with oxidative and energy-producing reactions may cause the release of potassium because this cannot be utilized. This seems a more likely explanation than one postulating decreased enzyme activity due to a fall in the intracellular potassium level. It may also explain the delay in onset of the reserpine-induced increase in $^{42}K_b$. That is to say that existing stores of metabolites must be exhausted before the reserpine effect becomes manifest.

We are grateful to Dr. C. Dale Falconer, of Ciba Laboratories Ltd., for generous supplies of reserpine and for help in other ways; to Mr. R. Callander for the graphs; to Dr. W. N. Holmes, of this department, for his help and advice in the isotope studies; and to Miss J. Hall for technical assistance.

REFERENCES

Barrett, W. E., Baker, T., and Plummer, A. J. (1956). *J. Pharmacol.*, **116**, 5.
Born, G. V. R. (1956). *J. Physiol.*, **131**, 704.
—— and Bülbring, E. (1956). Ibid., **131**, 690.
Fenn, W. O. (1940). *Physiol. Rev.*, **20**, 377.
Gillis, C. N., and Lewis, J. J. (1956a). *J. Pharm., Lond.*, **8**, 606.
—— —— (1956b). *Nature, Lond.*, **178**, 859.
—— —— (1957). Ibid., **179**, 820.
Hahn, L., and Hevesy, G. (1941). *Acta physiol. scand.*, **2**, 51.
Krebs, H. A., Eggleston, L. V., and Terner, C. (1951). *Biochem. J.*, **48**, 530.
Maizels, M. (1951). *J. Physiol.*, **112**, 59.
Noonan, T. R., Fenn, W. O., and Haege, L. (1941). *Amer. J. Physiol.*, **132**, 612.
O'Brien, J. M., and Wilde, W. S. (1952). *Science*, **116**, 193.
Sheppard, C. W. (1951). Ibid., **114**, 85.
West, T. C., Hadden, G., and Farah, A. (1951). *Amer. J. Physiol.*, **164**, 565.

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DETECTION AND MEASUREMENT OF ANTAGONISM TO FOLIC ACID

BY

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Methods of testing competitive antagonism in bacterial systems are considered and evaluated with particular reference to antifolic acids. Four antifolic acids—pteridine O/129, methotrexate, CB2295 and CB2335—were examined for competitiveness and potency in three test systems—*Leuc. citrovorum* and folinic acid, *Str. faecalis* and folinic acid, and *Str. faecalis* and pteroylglutamic acid. For comparison, phenol and formaldehyde were studied in the *Leuc. citrovorum* and folinic acid system. Interpretation of results was based on families of log. concentration/growth curves obtained in each system in the presence and absence of each antagonist. These curves were usually simple sigmoids. From these families of curves, *p*A values were derived and reciprocal growth/reciprocal concentration curves were drawn. Pteridine O/129 was fully competitive in all systems, while methotrexate was fully competitive in the *Str. faecalis* and folinic acid system, but did not give simple sigmoids in the other two systems. CB2295 showed some competitive features in all and CB2335 in the two systems in which it was active. It is concluded that the methods adopted, based on those used in vertebrate pharmacology, provide a stringent test of competitiveness in bacterial systems.

In screening potential antifolic acids (a term used here to refer to an antagonist of any vitamin of the folic acid group), Collier and Phillips (1954) and Timmis, Felton, Collier, and Huskinson (1957) employed a conventional microbiological method, which involved measuring growth of *Leuconostoc citrovorum* or *Streptococcus faecalis* at two or more levels of folic acid in the presence of varying amounts of antagonist. Though useful for screening, the method failed to provide a satisfactory measure of the degree of antagonism or competitiveness. To explore these properties further, we have developed from previous studies on antipurine action (Collier and Huskinson, 1957; Collier, 1957) a different method, which is the subject of the present paper. In this, we have measured growth of the test organism in response to geometrically increasing concentrations of folic acid, in the presence and absence of antagonist. The resulting log concentration/growth curves (CG curves) have been analysed by techniques used in vertebrate pharmacology (Gaddum, 1937; Schild, 1947; Chen and Russell, 1950; Ariëns, van Rossum, and Simonis, 1956).

MATERIALS AND METHODS

Four antifolic acids were studied: methotrexate (A-methopterin; Franklin, Belt, Stokstad and Jukes, 1949), 2:4-diamino-6:7-di*iso*propyl pteridine (O/129; Collier, Campbell and Fitzgerald, 1950), 2:4:6-triamino-5-benzeneazopyrimidine (CB2295; Timmis *et al.*, 1957) and 2:6-diamino-8-1′-naphthyl-8-azapurine (CB2335, Timmis *et al.*, 1957). Two non-specific inhibitors, formaldehyde *B.P.* and phenol *B.P.*, were also used.

Bacterial Growth.—Three systems were studied, namely, *Leuc. citrovorum* and folinic acid, *Str. faecalis* and folinic acid, and *Str. faecalis* and pteroylglutamic acid (PGA). Microbiological methods used were those described by Timmis *et al.* (1957), except that dilutions were arranged so that in any one test folinic acid or PGA was present in geometrically decreasing concentrations, while antagonist remained at constant concentration. Within a series of tests, the concentration of antagonist used was increased in geometric steps.

Analysis of CG Curves.—The basic unit of analysis was the family of curves obtained with one system and one antagonist. At least two replicate tubes were used in order to obtain any one point on a curve. Within a family, the mean maximal height of each curve obtained in the presence of antagonist was compared by a *t* test with that of the control curve obtained in its absence. In computing the mean maximal height all the appropriate replicates were used (see Figs. 1 and 2). "Parallelism" of each curve with the control was tested by regression

analysis after linearizing the central portions of the curves. The transformation used was

$$y' = \frac{y}{y_{max} - y}$$

where y is the response in ml. 0.1 N NaOH at a given concentration (x) in ng./ml. folic acid, and y_{max} is the maximum response obtained, since Gaddum (1937) showed that a plot of log. y′ against log. x was linear for the central portion of sigmoid dose-response curves. Constancy of interspace (that is, horizontal distance) between successive pairs of curves obtained in presence of antagonist was tested by deriving mathematically the molar ratios of antagonist to agonist at half-maximal control response, which is the point of minimum error.

In addition, CG curves were examined by the graphical method of Woolf (see Haldane, 1957) and of Lineweaver and Burk (1934), in which reciprocal of dose of agonist is plotted against reciprocal of response for each curve in a family (reciprocal concentration/reciprocal growth curves, RCG curves).

Degree of antagonism may be expressed as the molar ratio described above or as a *p*A value (Schild, 1947). In a particular family of transformed curves, *p*A values were obtained from those curves that had passed the test for parallelism with the control. The horizontal shifts from the control line in log. concentration of folic acid were plotted against negative log. molar concentration of antagonist (log shift curves). Straight lines were fitted by eye and, from these, pA_2 and pA_{10} values were read off by interpolation or extrapolation.

RESULTS

Leuc. citrovorum *and Folinic Acid.*—In this system, CB2335 was inactive. The families of CG curves obtained with O/129, methotrexate, CB2295, phenol and formaldehyde are shown in Figs. 1 to 5, the protocols for O/129 being given in Table I. With the exception of two curves obtained in the presence of higher concentrations of methotrexate, all curves in this system were simple sigmoids. In the two compound curves a break, significant in the regression analysis, occurred at a concentration of 0.625 ng./ml. folinic acid.

The families of curves illustrated in Figs. 1 to 5, including both sections of the two compound curves, were analysed by the methods described above. This analysis (Table II) showed that all curves obtained with O/129 were parallel with the control and of the same height. The molar ratios of antagonist to agonist and the interspace were constant. With methotrexate, the simple curve and the total compound curves were similar in height to the control, and all except part of one compound curve were parallel. The molar ratios determined at concentrations <0.625 ng./ml. folinic acid were not constant, but fell in a way consistent with a geometric series, and the interspace was constant. With CB2295 the CG curves were parallel to and of the same height as the control, but the molar ratios were neither constant nor in geometric series, nor was the interspace constant. With phenol and still more with formaldehyde, curves were vertically depressed and not parallel

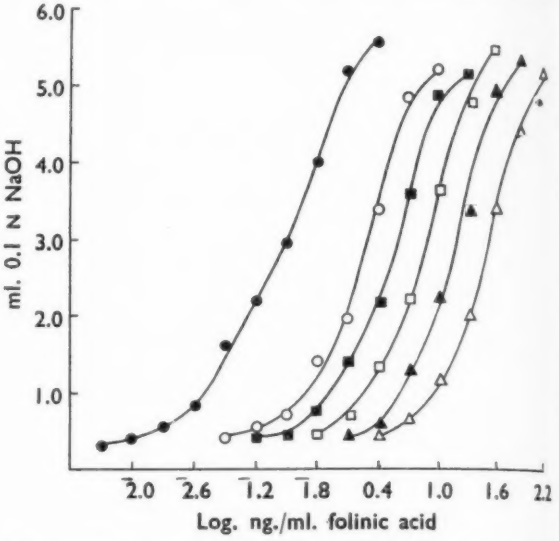

FIG. 1.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of O/129. ●——●, No antagonist; ○——○, 0.078 μg./ml. O/129; ■——■, 0.156 μg./ml. O/129; □——□, 0.312 μg./ml. O/129; ▲——▲, 0.624 μg./ml. O/129; △——△, 1.248 μg./ml. O/129.

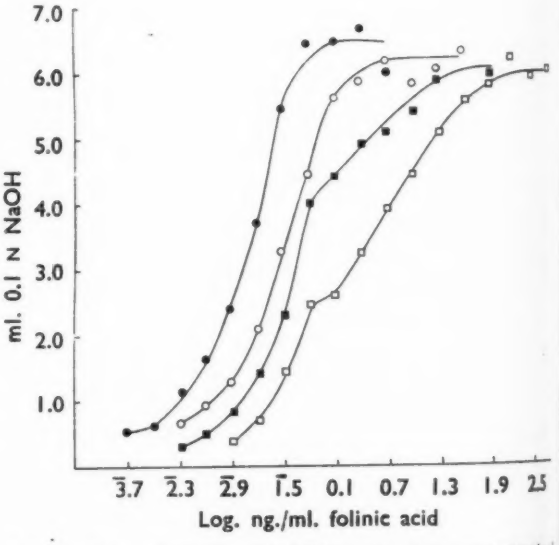

FIG. 2.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of methotrexate. ●——●, No antagonist; ○——○, 0.078 μg./ml. methotrexate; ■——■, 0.312 μg./ml. methotrexate; □——□, 1.248 μg./ml. methotrexate.

TABLE I

RESPONSE OF *LEUC. CITROVORUM* TO FOLINIC ACID IN THE PRESENCE AND ABSENCE OF O/129

Titres in ml. 0·1 N NaOH

μg./ml. O/129	ng./ml. Folinic Acid																
	160	80	40	20	10	5	2·5	1·25	0·625	0·3125	0·156	0·078	0·039	0·0195	0·0097	0·0048	0
1·248	5·4 4·9	4·6 4·2	3·9 2·9	1·8 2·2	0·9 1·3	0·7 0·6	0·4 0·5	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4						0·4 0·4
0·624		5·3 5·4	5·0 4·9	3·1 3·6	2·3 2·1	1·3 1·2	0·5 0·6	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4					0·4 0·4
0·312			5·5 5·4	4·6 5·0	3·6 3·7	2·0 2·4	1·3 1·3	0·6 0·8	0·4 0·5	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4				0·4 0·4
0·156				5·3 5·0	4·7 5·0	3·8 3·4	2·1 2·2	1·2 1·6	0·8 0·7	0·5 0·4	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4			0·4 0·4
0·078					5·7 4·7	5·0 4·7	3·7 3·1	2·1 1·8	1·4 1·4	0·7 0·7	0·6 0·5	0·4 0·4	0·4 0·4	0·4 0·4	0·4 0·4		0·4 0·4
0							5·8 5·3	5·1 5·3	3·8 4·2	3·0 2·9	2·3 2·1	1·7 1·5	0·8 0·9	0·6 0·5	0·5 0·5	0·4 0·4	0·4 0·4

TABLE II

LEUC. CITROVORUM AND FOLINIC ACID. ANALYSIS OF FAMILIES OF CONCENTRATION/GROWTH CURVES OBTAINED WITH O/129, METHOTREXATE, CB2295, PHENOL AND FORMALDEHYDE

— = Not tested. In the column headed Parallelism, + = no significant deviation from parallelism, $P > 0.05$; 0 = significant deviation from parallelism, $P < 0.05$.

Antagonist	Conc. of Antagonist (μg./ml.)	Slope		Height				Molar Ratio
		b	95% Fiducial Limits to b	Parallelism	% Control Maximum	Significance of Drop in Height		
						t	*P*	
O/129 ..	0	0·967	0·850–1·086	—	100·0	—	—	—
	0·078	1·050	0·847–1·254	+	90·7	1·36	>0·1	78·7
	0·156	1·190	1·046–1·333	+	90·1	2·30	>0·05	78·9
	0·312	1·227	1·041–1·412	+	98·2	0·39	>0·4	79·1
	0·624	1·200	1·050–1·350	+	96·4	0·78	>0·2	79·2
	1·248	1·158	1·086–1·230	+	92·8	1·13	>0·1	75·8
Methotrexate	0	1·060	0·718–1·402	—	100·0	—	—	—
	0·078	0·934	0·826–1·041	+	96·9	0·79	>0·2	95·2
	0·312	1·104	1·025–1·183	+	—	—	—	190·8
	0·312	0·563	0·140–0·986	—	94·8	0·96	>0·1	—
	1·248	1·131	0·926–1·337	+	—	—	—	382·4
	1·248	0·506	0·478–0·536	0	95·1	0·71	>0·2	—
CB2295 ..	0	1·117	0·819–1·415	—	100·0	—	—	—
	0·195	1·081	0·967–1·194	+	111·5	—	—	868
	0·780	1·099	0·987–1·211	+	106·1	—	—	1,741
	3·120	1·211	1·102–1·320	+	96·2	0·86	>0·2	1,389
	12·480	2·222	1·203–3·242	+	—	—	—	2,123
Phenol ..	0	1·994	1·817–2·172	—	100·0	—	—	—
	2,000	1·539	1·380–1·697	0	89·5	3·46	<0·01	—
	2,500	2·094	1·890–2·297	+	52·9	—	—	—
	3,125	—	—	—	0	—	—	—
Formaldehyde	0	0·983	0·837–1·129	—	100·0	—	—	—
	57·1	0·754	0·636–0·873	+	66·2	11·42	<0·001	—
	61·5	—	—	—	48·6	—	—	—
	66·7	—	—	—	25·7	—	—	—
	72·7	—	—	—	8·7	—	—	—
	100·0	—	—	—	0	—	—	—

TABLE III

*p*A VALUES FOR SOME ANTIFOLIC ACIDS IN THREE TEST SYSTEMS

Bacterial Species	Growth Factor	O/129			Methotrexate			CB2295			CB2335		
		pA_2	pA_{10}	pA_2-pA_{10}	pA_2	pA_{10}	pA_2-pA_{10}	pA_2	pA_{10}	pA_2-pA_{10}	pA_2	pA_{10}	pA_2-pA_{10}
Leuc. citrovorum	Folinic acid	7·02	6·35	0·67	6·85	5·47	1·38	6·11	5·40	0·71	—	—	—
		6·64	6·04	0·60	9·82	9·42	0·40	6·20	5·94	0·26	6·98	6·57	0·41
Str. faecalis	PGA	8·38	8·02	0·36	10·69	9·83	0·86	6·62	6·10	0·52	8·02	7·36	0·66

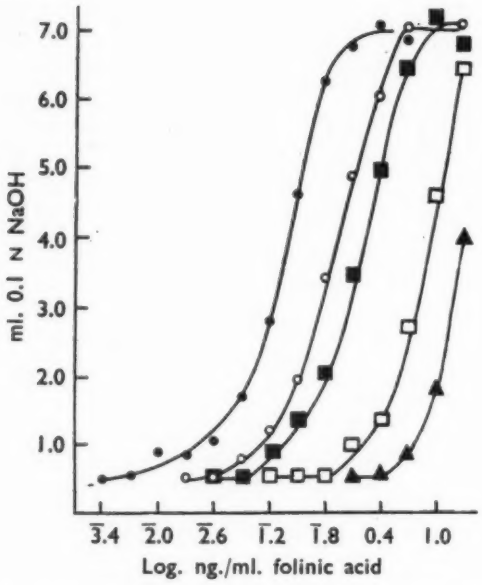

FIG. 3.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of CB2295. ●——●, No antagonist; ○——○, 0.195 μg./ml. CB2295; ■——■, 0.78 μg./ml. CB2295; □——□, 3.12 μg./ml. CB2295; ▲——▲, 12.48 μg./ml. CB2295.

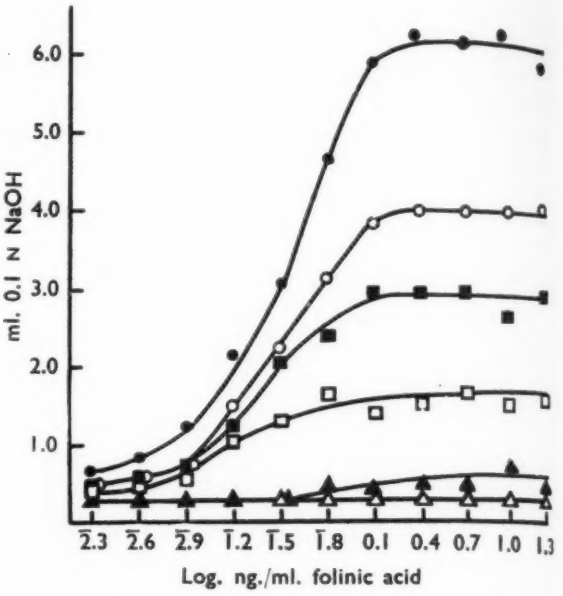

FIG. 5.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of formaldehyde. ●——●, No antagonist; ○——○, 57.1 μg./ml. formaldehyde; ■——■, 61.5 μg./ml. formaldehyde; □——□, 66.7 μg./ml. formaldehyde, ▲——▲, 72.7 μg./ml. formaldehyde; △——△, 100 μg./ml. formaldehyde.

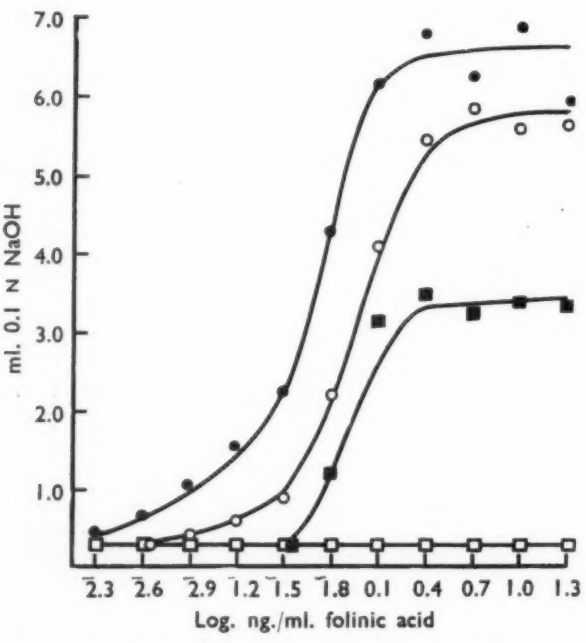

FIG. 4.—*Leuc. citrovorum* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of phenol. ●——●, No antagonist; ○——○, 2,000 μg./ml. phenol; ■——■, 2,500 μg./ml. phenol; □——□, 3,125 μg./ml. phenol.

with controls. The interpretation of these results in relation to competitiveness will be discussed below.

In the family of RCG curves obtained for O/129 (Fig. 6) by the method of Lineweaver and Burk (1934), the six lines met at the ordinate. CB2295 presented a similar picture. With formaldehyde (Fig. 7) we obtained a series of almost parallel lines. The figure obtained with phenol was essentially similar to that of formaldehyde, but the lines showed a greater change in slope.

Fig. 8 gives the log. shift curves for O/129, CB2295 and for methotrexate at concentrations <0.625 ng./ml. folinic acid. From such curves are derived the *p*A values summarized in Table III.

Str. faecalis *and Folinic Acid.*—The curves obtained with methotrexate and CB2335 (Figs. 9 and 10) were simple sigmoids. So also were those with CB2295 and O/129, which are not illustrated. The analysis of all four families is presented in Table IV. All curves passed the test for parallelism and for similarity of height, compared with their respective controls. With methotrexate the molar ratios did not vary appreciably, with O/129 they increased in a way consistent with a geometrical series, and with CB2295 and CB2335

these ratios were irregular. RCG curves were drawn for all four antifolic acids in this system and did not differ in type from Fig. 6. The *p*A values in Table III are derived from log. shift curves similar to those illustrated in Fig. 8.

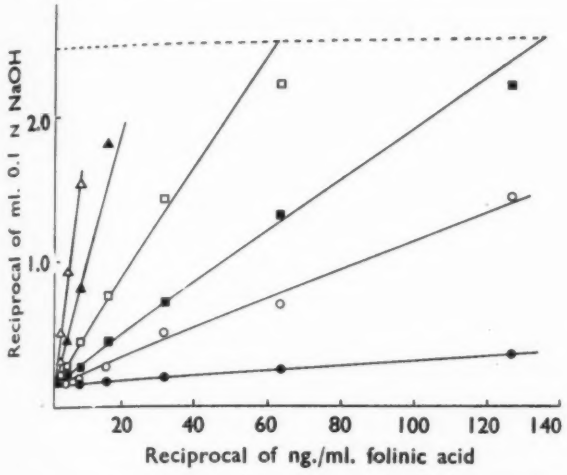

FIG. 6.—*Leuc. citrovorum* and folinic acid. Reciprocal concentration/reciprocal growth curves obtained in the presence and absence of O/129. Symbols as Fig. 1. The broken line indicates a value of 1 tube blank.

Str. *faecalis and PGA.*—O/129, CB2295 (Fig. 11) and CB2335 gave families of typical sigmoid curves analysed in Table V. Most CG curves passed the test for parallelism with their controls, but all families showed a drop in height at high concentrations of antagonist. The molar ratios for O/129 and CB2335 decreased apparently geometrically and those for CB2295 were irregular. RCG curves for these three substances resembled Fig. 6. Their *p*A values (Table III) were derived from log. shift curves similar to those illustrated in Fig. 8.

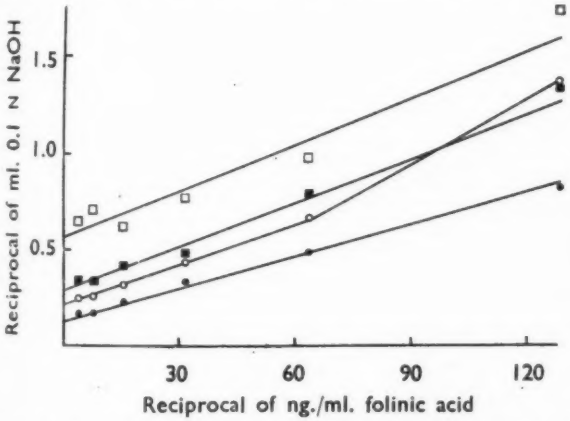

FIG. 7.—*Leuc. citrovorum* and folinic acid. Reciprocal concentration/reciprocal growth curves obtained in the presence and absence of formaldehyde. Symbols as Fig. 5.

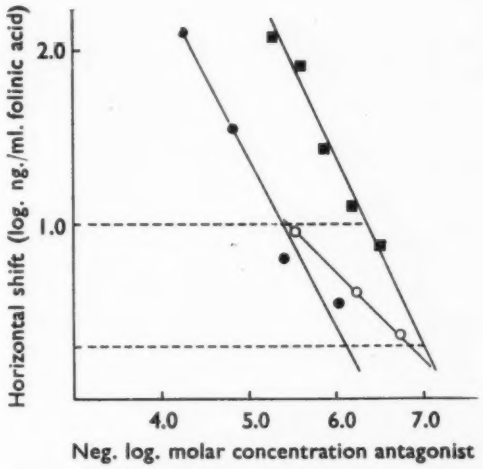

FIG. 8.—*Leuc. citrovorum* and folinic acid. Log. shift/neg. log. molar concentration curves obtained with antifolic acids and estimation of pA_x values. The broken lines intersect the ordinate at log 10 and log 2; their intercepts with the curves are at points corresponding on the abscissa to the pA_{10} and pA_2 values. ●———●, CB2295; ○———○, methotrexate; ■———■, O/129.

The curves of methotrexate (Fig. 12) differed from those of the above substances in several features. All were compound, showing two maxima, and portions of the curves obtained at 0.078 and 0.312 ng./ml. methotrexate fell to the left of the control line. Except for these two portions, each part of the curves was tested separately for parallelism. The curve at 0.312 ng./ml. was not tested for height, because we thought that it was incomplete; but all other compound curves were not significantly lower than the control. The log. shifts were obtained from the second ascending portions of the compound curves at half-maximal control growth, and the *p*A values thus derived are given in Table III.

DISCUSSION

The fundamental concept of competitive interaction rests on competition between two substances for one receptor system. Competitive antagonism occurs where one of these substances has little or no intrinsic activity (Ariëns *et al.*, 1956). Applying this definition, it can be shown that with a competitive antagonist CG curves should be parallel to the control and of constant height. Ariëns *et al.* have shown that, for geometrically increasing doses of antagonist, the interspace between successive CG curves obtained in the presence of a competitive antagonist should be constant. Where the same numbers of mole-

TABLE IV

STR. FAECALIS AND FOLINIC ACID. ANALYSIS OF FAMILIES OF CONCENTRATION/GROWTH CURVES OBTAINED WITH O/129, METHOTREXATE, CB2295, AND CB2335

See Table II for explanation of symbols in column headed Parallelism.

Antagonist	Conc. of Antagonist (ng./ml.)	Slope			Height			Molar Ratio
		b	95% Fiducial Limits to b	Parallelism	% Control Maximum	Significance of Drop in Height		
						t	*P*	
O/129 ..	0	1·083	0·957–1·211	—	100·0	—	—	—
	78	0·978	0·738–1·218	+	99·0	0·149	>0·5	469
	156	1·124	1·022–1·227	+	96·3	0·795	>0·2	505
	312	1·116	1·040–1·193	+	99·0	0·149	>0·5	568
Methotrexate	0	0·901	0·763–1·040	—	100·0	—	—	—
	0·0975	0·973	0·694–1·607	+	96·5	1·029	>0·1	0·506
	0·390	1·061	0·725–1·397	+	97·6	0·611	>0·2	0·535
	1·560	0·945	0·724–1·166	+	99·5	—	—	0·563
CB2295 ..	0	0·976	0·672–1·279	—	100·0	—	—	—
	195	0·820	0·596–1·045	+	107·5	—	—	901
	780	0·850	0·573–0·948	+	93·2	1·417	>0·1	1,193
	3,120	0·738	0·573–0·903	+	97·4	0·359	>0·3	689
CB2335 ..	0	0·942	0·830–1·053	—	100·0	—	—	—
	15·6	0·998	0·762–1·233	+	98·2	0·447	>0·5	464
	62·5	1·019	0·839–1·199	+	97·6	0·518	>0·3	733
	250	1·004	0·774–1·232	+	95·6	0·660	>0·2	733
	1,000	0·968	0·793–1·141	+	101·0	—	—	915

cules of agonist and of antagonist can combine with one receptor unit, constancy of interspace is shown by a constant molar ratio of agonist to antagonist. Where the numbers of competing molecules are unequal, the interspace remains constant but the molar ratios lie in a geometric series.

When the interspace between CG curves is constant, the slope of the corresponding RCG curves increases constantly. Even if the interspace is not constant, provided the CG curves are parallel with their control and of the same height, the lines of the RCG plot will intersect on the ordinate, although their increase in slope will not be constant.

When the interspace between CG curves is constant, the lines obtained in log. shift plots will be

TABLE V

STR. FAECALIS AND PTEROYLGLUTAMIC ACID. ANALYSIS OF FAMILIES OF CONCENTRATION/GROWTH CURVES OBTAINED WITH O/129, METHOTREXATE, CB2295 AND CB2335

See Table II for explanation of symbols in column headed Parallelism.

Antagonist	Conc. of Antagonist (ng./ml.)	Slope			Height			Molar Ratio
		b	95% Fiducial Limits to b	Parallelism	% Control Maximum	Significance of Drop in Height		
						t	*P*	
O/129 ..	0	1·354	1·136–1·553	—	100·0	—	—	—
	1·25	1·134	0·873–1·394	+	95·1	1·679	>0·05	2·57
	2·5	1·227	1·110–1·344	+	94·8	1·860	>0·05	1·82
	5·0	0·805	0·699–0·912	0	73·1	9·589	<0·001	1·24
	10·0	1·063	0·661–1·466	+	77·0	5·158	<0·001	0·80
Methotrexate	0	1·190	0·900–1·480	—	100·0	—	—	—
	0·078	1·156	0·703–1·609	+	100·2	—	—	0·132
	0·312	0·686	0·468–0·903	+	—	—	—	0·047
	1·248	0·994	0·273–1·715	+	—	—	—	—
	1·248	2·654	1·873–3·435	0	107·1	—	—	0·035
	4·992	2·049	1·575–2·524	0	—	—	—	—
	4·992	1·965	1·239–2·691	+	106·8	—	—	0·031
CB2295 ..	0	1·386	0·972–1·799	—	100·0	—	—	—
	78	0·753	0·510–0·996	+	97·3	1·549	>0·05	300
	312	0·908	0·625–1·190	+	96·0	1·842	>0·05	338
	1,248	0·894	0·501–1·288	+	93·1	3·750	<0·01	170
	4,992	—	—	—	32·0	—	—	—
CB2335 ..	0	1·278	1·118–1·437	—	100·0	—	—	—
	9·75	1·540	1·369–1·711	+	100·0	—	—	13·1
	19·5	1·007	0·818–1·195	+	90·1	4·76	<0·001	12·4
	39·0	1·445	0·961–1·929	+	95·0	2·95	<0·01	11·6
	156·0	—	—	—	31·1	—	—	—

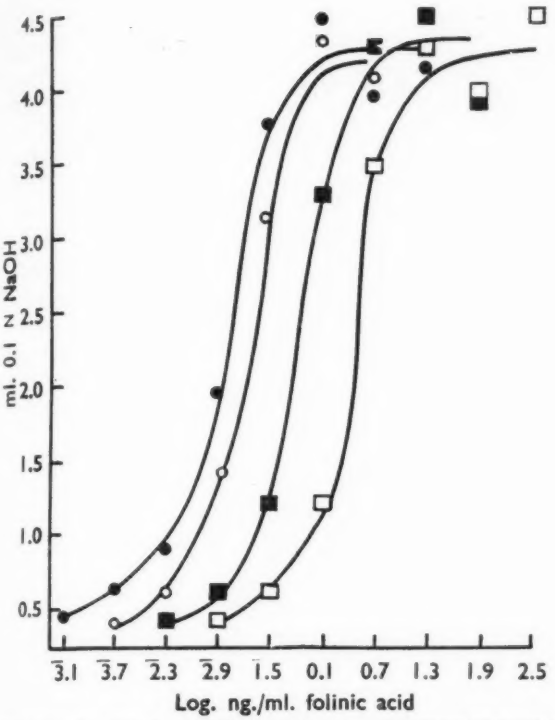

FIG. 9.—*Str. faecalis* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of methotrexate. ●——●, No antagonist; ○——○, 0.0975 ng./ml. methotrexate; ■——■, 0.390 ng./ml. methotrexate; □——□, 1.560 ng./ml. methotrexate.

rectilinear. Where this condition did not hold, the best straight lines were fitted by eye, in order to obtain *p*A values (Fig. 8). Schild (1947) has shown from the mass action equation of Gaddum (1937) that " a first order reaction requires a ninefold increase of antagonist corresponding to a five-

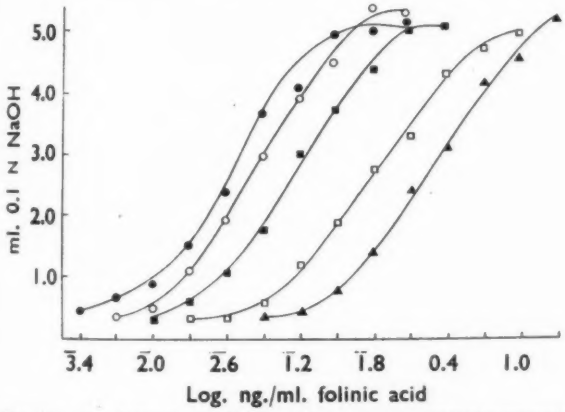

FIG. 10.—*Str. faecalis* and folinic acid. Log. concentration/growth curves obtained in the presence and absence of CB2335. ●——●, No antagonist; ○——○, 62.5 ng./ml. CB2335; ■——■, 250 ng./ml. CB2335; □——□, 1,000 ng./ml. CB2335.

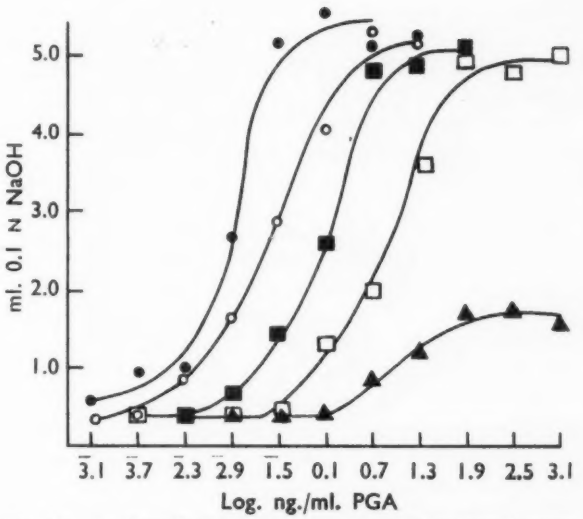

FIG. 11.—*Str. faecalis* and PGA. Log. concentration/growth curves obtained in the presence and absence of CB2295. ●——●, No antagonist; ○——○, 78 ng./ml. CB2295; ■——■, 312 ng./ml. CB2295; □——□, 1,248 ng./ml. CB2295; ▲——▲, 4,992 ng./ml. CB2295.

fold increase of active drug." This gives the equation

$$pA_2 - pA_{10} = 0.95$$

It is clear, however, that the value of 0.95 will only be realized in a situation where one molecule of

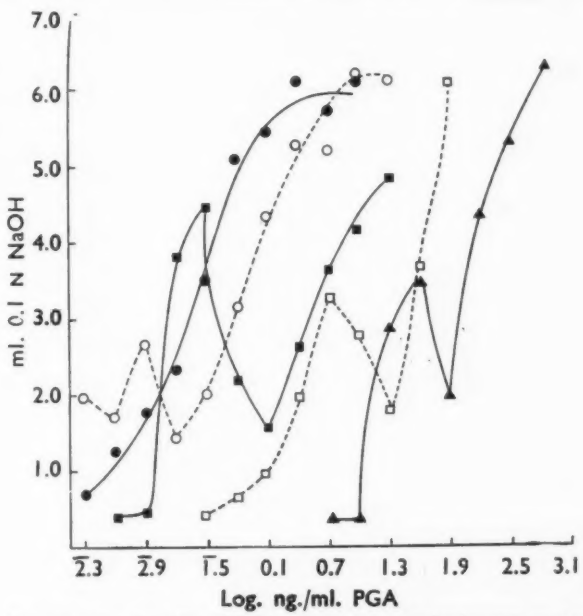

FIG. 12.—*Str. faecalis* and PGA. Log. concentration/growth curves obtained in the presence and absence of methotrexate. ●——●, No antagonist; ○------○, 0.078 ng./ml. methotrexate; ■——■, 0.312 ng./ml. methotrexate. □-----□, 1.248 ng./ml. methotrexate; ▲——▲, 4.992 ng./ml. methotrexate.

agonist or one of antagonist combine with one receptor unit. Marshall (1955) found that atropine and acetylcholine gave a $pA_2 - pA_{10}$ value of 0.73 on guinea-pig ileum. Although he inferred from this that their relationship was not competitive, this need not be so, as Timms (1956) points out and the present discussion makes clear.

Of the compounds examined, only O/129 fulfilled in all three systems the criteria of competitive antagonism discussed above. The $pA_2 - pA_{10}$ values, however, were less than 0.95, which may be explained by supposing that the relationships between O/129 and folic acid with the receptors are not of the first order.

In the *Str. faecalis* and folinic acid system, methotrexate fulfilled the criteria of competitive antagonism. In both the other systems, however, it presented a complicated picture. With *Leuc. citrovorum* and folinic acid, breaks appeared in some of the CG curves, although corresponding effects were not detected with the earlier method of investigation (Collier and Phillips, 1954). It was possible, however, to obtain *p*A values from the log shifts of the parallel portions of the curves. The $pA_2 - pA_{10}$ value was much greater than unity, which was exceptional. In the *Str. faecalis* and PGA system, methotrexate at low concentrations slightly stimulated growth (Fig. 12), which suggests that it acts at more than one of the receptors concerned with the utilization of PGA. This may help to account for the unusual shape of the CG curves.

CB2295 and CB2335 exhibit some competitive features, but generally lack that of constant interspace over the concentration ranges tested. The two compounds are clearly antifolic acids, which may well be fully competitive over some ranges of concentration. The drop in the maxima of the CG curves at high concentrations seen with CB2295, CB2335, and O/129 in some systems indicates toxicity unconnected with folic acid.

Phenol shows some degree of parallelism, but no other competitive features. Formaldehyde provides a series of curves of decreasing maxima without horizontal shift, except for a slight initial one. It thus exemplifies inhibition that is not competitive.

Our findings show that the technique of testing competitiveness developed in vertebrate pharmacology can be applied to bacterial systems. They emphasize the necessity for full tests of this type before relationships can be characterized as competitive. They also show that chemical structure and potency can be guides to competitive action. Thus, both compounds which were fully competitive in at least one system (O/129 and methotrexate) are pteridines and were also the most potent inhibitors tested. CB2295 and CB2335, which show some features of competitiveness, are pyrimidines and were of moderate potency. Phenol and formaldehyde, which were not competitive, do not resemble folic acid in structure and were of relatively low potency.

We wish to thank Miss J. A. Moore and Miss J. Wallace for technical assistance. We are indebted to Professor F. C. Happold for the culture of *Str. faecalis* (R) and to the American Cyanamid Company for methotrexate ("A-methopterin") and the calcium salt of folinic acid ("Calcium leucovorin").

References

Ariëns, E. J., van Rossum, J. M., and Simonis, A. M. (1956). *Arzneim. Forsch.*, **6**, 282.

Chen, G., and Russell, D. (1950). *J. Pharmacol.*, **99**, 401.

Collier, H. O. J. (1957). *Pharmacol. Rev.*, **9**, 264.

—— Campbell, N. R., and Fitzgerald, M. E. H. (1950). *Nature, Lond.*, **165**, 1004.

—— and Huskinson, P. L. (1957). *Ciba Foundation. Symposium on the Chemistry and Biology of Purines*, p. 146. London: Churchill.

—— and Phillips, M. (1954). *Nature, Lond.*, **174**, 180.

Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H. (1949). *J. biol. Chem.*, **177**, 621.

Gaddum, J. H. (1937). *J. Physiol.*, **89**, 7P.

Lineweaver, H., and Burk, D. J. (1934). *J. Amer. chem. Soc.*, **56**, 658.

Marshall, P. B. (1955). *Brit. J. Pharmacol.*, **10**, 354.

Schild, H. O. (1947). Ibid., **2**, 189.

Timmis, G. M., Felton, D. G. I., Collier, H. O. J., and Huskinson, P. L. (1957). *J. Pharm., Lond.*, **9**, 46.

Timms, A. R. (1956). *Brit. J. Pharmacol.*, **11**, 273.

Woolf. B. (1932). See Haldane, J. B. S. (1957). *Nature, Lond.*, **179**, 832.

Brit. J. Pharmacol. (1957), **12**, 529.

INTRAVENTRICULAR ADMINISTRATION OF RESERPINE AND ITS METABOLITES TO CONSCIOUS CATS

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Intraventricular injection of reserpine in conscious cats produced relaxation of the nictitating membrane, miosis with narrowing of the palpebral fissure to a slit, avoidance of light, diarrhoea, anorexia, and tranquillization. Equivalent doses of trimethoxybenzoic acid, reserpic acid or methylreserpate separately or in combination did not produce such effects, indicating that these reserpine metabolites were not involved in these reserpine actions. 5-Hydroxytryptamine alone or in combination with subthreshold doses of reserpine also did not duplicate the effects observed after effective doses of reserpine. The possible central sites of reserpine action are discussed.

In a recent review Bein (1956) suggested that reserpine may not act *per se*, but may be converted into an active substance in the tissues, or release an active substance (5-hydroxytryptamine), or act only in concert with the latter. The first suggestion was supported by studies on the metabolism of ^{14}C-labelled reserpine which indicated that very little reserpine was localized in the brain whereas much higher concentrations of its metabolites were present (Numeroff, Gordon, and Kelly, 1955 ; Sheppard, Lucas, and Tsien, 1955 ; Glazko, Dill, Wolf, and Kazenko, 1956). Further support was obtained when Gaddum and Vogt (1956) showed that 10 μg. of reserpine intraventricularly had no effect, and Sturtevant and Drill (1956) reported that 100 μg. intraventricularly caused an autonomic nervous system discharge followed by a typical reserpine tranquillization 24 hr. later. However, these doses may have been either too small or too large to induce typical reserpine effects within a time interval approaching that observed following other routes of administration. Furthermore, Peets and Schulert (1957) recently found that totally labelled ^{14}C-reserpine reached its highest brain concentration within 20 min. Plummer, Sheppard, and Schulert (1957), using tritium-labelled reserpine, showed a significant concentration of the drug in the brain for periods up to 48 hr. Plummer *et al.* (1957), therefore, suggested that reserpine might act *per se* and not through an indirect mechanism. In an attempt to elucidate the problem, we have studied the effects produced in conscious cats by the intraventricular injection of reserpine and its metabolites, as well as 5-hydroxytryptamine.

METHODS

Five cats had cannulae implanted in the right lateral ventricle according to Feldberg and Sherwood (1953). Studies were made on the behavioural and other changes produced when the drugs were administered in saline solutions in a total volume of 0.2 ml. When required, the *p*H of the solutions was adjusted to 7.0. All doses of the following drugs refer to the free acid or base: reserpine phosphate, trimethoxybenzoic acid, reserpic acid, methylreserpate, and 5-hydroxytryptamine creatinine sulphate (5-HT). All drugs except the last were studied by the blind technique and another independent observer always checked the observations of the authors. Observations covered a period of 6 to 8 hr. after injection, and drugs were given only once a week.

RESULTS

Reserpine.—The intraventricular injection of 10 to 16 μg. of reserpine produced no detectable behavioural or other effects in the cats. This confirmed the observation of Gaddum and Vogt (1956). After a latent period of 30 min., 18 to 20 μg. of reserpine caused relaxation of the nictitating membrane, miosis with narrowing of the palpebral fissure to a slit, squinting in response to light, withdrawal to the darkest corner of the cage, diarrhoea, anorexia, tranquillization, and a pronounced generalized depression. The anorexia was still present the following day, but the other effects appeared to be terminated in 24 hr. When

the dose of reserpine was increased to 40 μg., the same responses were obtained, but the latent period preceding their onset was reduced to 20 min. With both doses, normally aggressive cats became docile and fearful cats became friendly. In one experiment in which there was a high noise level, 40 μg. of reserpine produced no tranquillization and the cats remained alert. These observations agree with those of Bein (1956) but disagree with the findings of Sturtevant and Drill (1956). The differences are probably related to the 100 μg. dose of reserpine used by the latter investigators.

Reserpine Metabolites.—Intraventricular injection of 20 μg. of trimethoxybenzoic acid caused no reaction. Increasing the dose to 40 μg. resulted in decreased alertness and a slight anorexia. This latter effect differed from that produced by reserpine, because the animals would eat if encouraged to do so.

Intraventricular injection of 20 μg. of reserpic acid produced only defaecation and the animals appeared normal and consumed their usual quantity of food. Increasing the dose of reserpic acid to 40 μg. caused a decreased alertness but no tranquillization. One cat developed convulsive seizures affecting the left side 57 min. after injection. These seizures were of 30 sec. duration in the beginning but became continuous after 1.5 hr. They could be induced by loud noises. During the seizures the cat had a vacant stare, mydriasis, piloerection and howled continuously. Intraperitoneal injection of 30 mg./kg. of sodium pentobarbitone anaesthetized the cat and terminated the convulsions.

Intraventricular administration of 20 to 40 μg. of methylreserpate produced no effects in the cats other than a slight decrease in alertness.

When mixtures containing 20 μg. and 40 μg. each of trimethoxybenzoic acid, reserpic acid and methylreserpate were given, there were no significant changes in the cats.

5-Hydroxytryptamine.—Intraventricular injection of 100 μg. of 5-HT produced effects similar to those described by Feldberg and Sherwood (1954) and Gaddum and Vogt (1956). Increasing the dose to 500 μg. increased the degree of sedation, but its duration was only 1 hr. There was no anorexia and the overall effects of 5-HT were not the same as those seen after reserpine. Gaddum and Vogt (1956) have reviewed the evidence concerning the actions of reserpine and their relationship to its known ability to release 5-HT in the brain. They pointed out both the similarities and dissimilarities between the two drugs and observed an increase in lethargy when 5-HT was given intraventricularly 16 hr. after reserpine. If the action of reserpine is based upon 5-HT release, it might be possible to duplicate the effects of the former by simultaneous intraventricular administration of subthreshold doses of both drugs. Administration of 10 μg. of reserpine and 100 μg. of 5-HT was entirely ineffective in duplicating the effects seen when either reserpine or 5-HT were given alone. However, one cat died of respiratory paralysis 4 hr. after receiving the mixture. Tonic seizures preceded death, but no definitive cause of death could be established at necropsy. Furthermore, such reactions were not observed in the other animals.

Discussion

The results presented here indicate that typical central effects ascribed to reserpine are produced by minute quantities of this drug *per se*, and they cannot be duplicated by its metabolites either alone or in combination. The central actions of 5-HT, although similar in certain aspects, are not entirely the same as those of reserpine and do not have a comparable duration of action. Furthermore, a combination of subthreshhold doses of both drugs given simultaneously does not duplicate the effects seen after either drug alone. Thus our results would tend to answer the questions posed by Bein (1956) and indicate, in agreement with Plummer *et al.* (1957), that the central effects seen after administration of reserpine are related to that drug *per se*. The time interval for the onset of effect agrees with that found by Peets and Schulert (1957), and is probably related to the interval required for the drug to move throughout the ventricular spaces, diffuse into the tissues and attach itself to receptors. There can be little doubt that the effects observed have their origin in central sites because of the mode of administration and the small quantity of reserpine required for their production. However, it is possible that not all of the effects are a result of direct drug-receptor combinations, because activation of remote sites through interneuronal connexions may take place under the conditions of our experiments. On the other hand, certain definitive sites must be implicated in producing the effects observed. Stimulation of the parasympathetic sites in the anterior hypothalamus would account for the relaxation of the nictitating membrane and the miosis. The defaecation could result from stimulation of the pre- and supra-optic nuclear areas of the hypothalamus (Sheenan, 1940). The tranquillization and generalized depression probably involve the ascending

reticular formation with its collaterals in the hypothalamus because the area has been shown to be concerned with states of consciousness (Magoun, 1951–2). Moreover, our results support the suggestion of Schneider and Earl (1954) that changes have been produced in the central regulating mechanism of the autonomic nervous system in the brain stem.

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References

Bein, H. J. (1956). *Pharmacol. Rev.*, **8**, 435.
Feldberg, W., and Sherwood, S. L. (1953). *J. Physiol.*, **120**, 3P.
—— —— (1954). Ibid., **123**, 148.
Gaddum, J. H., and Vogt, M. (1956). *Brit. J. Pharmacol.*, **11**, 175.
Glazko, A. J., Dill, W. A., Wolf, L. M., and Kazenko, A. (1956). *J. Pharmacol.*, **118**, 377.
Magoun, H. C. (1951–52). *An Ascending Reticular System in the Brain Stem, The Harvey Lectures*, p. 53. New York: Academic Press, Inc.
Numeroff, P., Gordon, M., and Kelly, J. M. (1953). *J. Pharmacol.*, **115**, 427.
Peets, E. A., and Schulert, A. R. (1957). *Abst. of Papers, Div. of Biol. Chem., Amer. Chem. Soc., Miami, Florida, April* 7–12, p. 70C.
Plummer, A. J., Sheppard, H., and Schulert, A. R. (1957). *Abst. Int. Symposium on Psychotropic Drugs, Milan, Italy, May* 9–11, p. 50.
Schneider, J. A., and Earl, A. E. (1954). *Neurology*, **4**, 657.
Sheenan, D. (1940). *Res. Publ. Ass. Research Nervous Mental Disease*, **20**, 589.
Sheppard, H., Lucas, R. C., and Tsien, W. H. (1955). *Arch. int. Pharmacodyn.*, **103**, 256.
Sturtevant, F. M., and Drill, V. A. (1956). *Anat. Rec.*, **125**, 607.

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THE CONCENTRATION OF ADRENALINE IN THE PLASMA OF RABBITS TREATED WITH RESERPINE

BY

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Doses of 1 to 2.3 mg./kg. of reserpine given intravenously to rabbits raised the plasma adrenaline. The noradrenaline concentrations were too near the threshold of the method to decide whether any alterations followed the injection of reserpine. Control experiments, in which blood samples were taken from rabbits but no drug was given, showed only a very slight rise in adrenaline concentration after the first bleeding.

It is known that reserpine causes a loss of adrenaline and noradrenaline from the adrenal medulla (Holzbauer and Vogt, 1956 ; Carlsson and Hillarp, 1956), from the brain (Holzbauer and Vogt, 1956 ; Shore, Olin, and Brodie, 1957), from the heart (Bertler, Carlsson, and Rosengren, 1956 ; Paasonen and Krayer, 1957), and from peripheral sympathetic tissue (Muscholl and Vogt, 1957a and b). The animal which has received reserpine, however, does not exhibit very obvious signs of increased sympathetic activity such as would be expected during a discharge of adrenal medullary amines. In fact, the well-known signs of miosis and relaxation of the nictitating membrane produced by injections of reserpine have usually been interpreted as signs of diminished sympathetic activity. The question might therefore be asked whether the amines lost from the tissues are really released into the circulation or whether the losses are caused by inhibition of amine synthesis. There are several observations which favour the view that there is genuine release of amines after an injection of reserpine. Everett, Toman, and Smith (1957) have shown that, 30 min. after a very large dose of reserpine, mice pass through a phase of piloerection which is followed by a long period during which the piloerector reflex to cold is abolished. Kuschke and Frantz (1955) demonstrated a hyperglycaemic effect of reserpine in the rabbit which was sensitive to ergot alkaloids ; the effect was not abolished by splanchnotomy. Rises in blood pressure in the rat and the spinal dog, and contractions of the denervated nictitating membrane of the cat, have been reported to follow injections of reserpine (de Jongh and v. Proosdij-Hartzema, 1955 ; Maxwell, Ross, Plummer, and Sigg, 1957).

In order to see whether proof of an increase in circulating adrenaline could be obtained, direct estimations were carried out of the adrenaline concentration in the plasma of rabbits during the first 90 min. after an injection of reserpine. The rabbit was chosen because sufficient volumes of blood can be collected from a cut in the ear vein without restraining or anaesthetizing the animal. Further, in our experience, the medullary amines are more rapidly mobilized by reserpine from the adrenals of rabbits than from those of other laboratory animals. The disadvantage of the rabbit is the high 5-hydroxytryptamine (5-HT) content of its blood which greatly complicates the assay.

METHODS

Rabbits of an average weight of 2.9 kg. were used. The region of the marginal ear vein was shaved 1 or 2 days before the experiment. In order to collect blood, heparin (400 i.u./kg.) was injected into one ear vein and soon afterwards a small cut was made across the vein. The escaping blood was collected into an ice-cooled centrifuge tube containing 200 i.u. heparin. When 9 to 17 ml. of blood had been obtained, the haemorrhage was stopped by placing a light aluminium clip across the puncture. Reserpine was then injected into the vein of the other ear. Between 35 and 90 min. later, a second blood sample was collected by removing the clip and wiping the region of the puncture so that blood was again flowing freely from the vessel. With one exception, difficulty in obtaining blood owing to vasoconstriction was only experienced when iproniazid had been administered before the reserpine. Thus, after iproniazid and reserpine, the time required to obtain 6 ml. plasma (10 ml. blood) averaged 25 min., whereas the corresponding collection time after reserpine only was

4.8 min. In these experiments, and in one animal not given iproniazid, the ear had to be bathed repeatedly in warm saline in order to accelerate the flow.

The reserpine was given as Serpasil (Ciba) in doses of 1 to 2.3 mg./kg. Iproniazid (100 mg./kg. intravenously) was given either 1 or 14 hr. before the reserpine. Whereas rabbits not given any drugs showed no ill effects from the bleeding, all rabbits bled and injected with reserpine became comatose a few hours after the experiments and died in the course of the next 48 hr.

The cooled blood was centrifuged for 15 min. at 3,500 rev./min. and the plasma (usually between 6 and 9 ml.) was stirred into 6 vol. of cooled acid ethanol (0.1 ml. conc. HCl in 100 ml. ethanol). The further treatment of the alcoholic extract, the application to paper for separation of the amines by chromatography and the elution procedure were as previously described (Vogt, 1952).

The eluate of the noradrenaline region was tested on the blood pressure of the pithed rat. Rats of about 220 g. weight were injected subcutaneously with 1 to 2 mg. of atropine sulphate and deeply anaesthetized with ether. A tracheal cannula was then inserted and the central nervous system destroyed by introducing a wire (14 SWG) into one orbit, and passing it through the foramen magnum into the spinal canal (Shipley and Tilden, 1947). Artificial respiration was started immediately after the pithing. Such rats have a blood pressure between 50 and 60 mm. Hg and respond to the injection of 1 ng. of noradrenaline.

Adrenaline assays were carried out on the rat uterus stimulated by carbachol (Gaddum and Lembeck, 1949). On account of the large amounts of 5-HT present in the samples a special technique was required. The R_F value of 5-HT is only very slightly greater than that of adrenaline, so that eluates of the adrenaline region contain all the 5-HT recovered from the extracts. Before attempting the assay of adrenaline, the 5-HT equivalent of an aliquot of the eluate was determined on the rat uterus. Then a quantity of lysergic acid diethylamide (LSD) sufficient to abolish the effect of the highest 5-HT equivalent found in the eluates was added to the bath (doses of 0.15 to 1 μg. LSD were needed for a 2 ml. bath; they were left in the bath for 10, or occasionally 20, min.). The adrenaline was then assayed against standard solutions to which as much 5-HT had been added as had been found in the eluates. 0.05 or 0.1 ml. eluate corresponded to 1 ml. original plasma. Whenever adrenaline-like activity was found, an aliquot of the eluate was heated in a sealed tube at *p*H 8 to check for heat lability of the active substance.

Results

Adrenaline.—It is known that the administration of reserpine to animals, previously treated with the amine oxidase inhibitor iproniazid, causes excitement and increased sympathetic activity, and that, in cats and dogs, iproniazid converts the depressor effect of reserpine into a pressor response (Brodie, Pletscher, and Shore, 1956; Chessin, Dubnick, Kramer, and Scott, 1956; Besendorf and Pletscher, 1956). The first experiments were carried out with this combination of drugs in the belief that the destruction of circulating adrenaline might thus be retarded (see Table I, rabbits 1 to 3).

TABLE I

ADRENALINE CONCENTRATION IN THE PLASMA OF RABBITS BEFORE AND AFTER AN INTRAVENOUS INJECTION OF RESERPINE

The ear vessels of rabbit No. 7 had to be dilated by bathing in warm saline during the collection of the first blood sample (S_1). In rabbit No. 1, S_1 was obtained before giving iproniazid, but in rabbits Nos. 2 and 3, S_1 was measured after iproniazid.

No. of Rabbit	Reserpine (mg./kg.)	Iproniazid Before Reserpine	Adrenaline			Interval between Reserpine and S_2 (min.)
			μg./l. Plasma		% Rise	
			S_1 (Before Reserpine)	S_2 (After Reserpine)		
1	1·0	1 hr. before	<0·2	1·2	>500	43
2	2·2	14 hr. before	<0·8	3·0	>260	55
3	1·0	1 hr. before	1·3	2·0	54	80
4	2·3	None	0·1	1·2	1,100	38
5	2·3	,,	0·2	1·1	450	40
6	2·3	,,	<0·05	0·2	>300	40
7	2·2	,,	4·0	10·0	150	58
8	2·1	,,	0·8	1·6	100	97
9	None	,,	0·3	0·4	33	35
10	,,	,,	1·6	2·0	25	78

There was, in all three instances, an increase in circulating adrenaline after the reserpine. This was independent of the fact that rabbits Nos. 1 and 3, which had reserpine 1 hr. after iproniazid, were sedated, whereas rabbit No. 2, with a 14 hr. interval between the two drugs, was excited during the collection of sample 2. The experiments were, however, considered unsatisfactory because the intense vasoconstriction of the ear vessels slowed down the collection of the second samples and necessitated frequent bathing of the ear in warm saline and therefore much handling of the rabbit; this in itself might have increased the blood adrenaline.

The next five rabbits were treated with reserpine only, and these experiments were much more satisfactory. There was little difference in the collection time of the blood samples before and after reserpine, bathing of the ear with warm saline was not usually required, and the rabbits appeared indifferent to the procedure. All five rabbits (Table I, Nos. 4 to 8) had large increases in the adrenaline concentration of the blood after reserpine. The % increases appeared to be greater when the interval

between injection and sampling was short, but this may have been accidental, particularly since the absolute increases in circulating adrenaline did not reflect the same trend. It will be noted that the adrenaline concentrations before injecting the drug were very variable; rabbit No. 7, which had an abnormally high initial concentration, had constricted ear vessels and required bathing to obtain the requisite amount of blood. After reserpine the ear vessels were dilated, and the rabbit was sedated, easy to bleed, and no further warming of the ear was required. In spite of this, the adrenaline concentration was 2.5 times higher than before.

There was, however, one objection which could be raised against attributing the rise in circulating adrenaline to the action of reserpine. Haemorrhage is a physiological stimulus of the sympathetic system; thus it was conceivable that the blood loss connected with taking the first sample and not the drug was the cause of the increase in circulating adrenaline. Two experiments were therefore carried out on rabbits (Table I, Nos. 9 and 10) of the same size as those used before. Two blood samples were taken, the time allowed between the two samples covering the same range as the interval in the experiments with reserpine. The adrenaline content of sample 2 was, indeed, slightly higher than that of sample 1, but the increase was very much smaller than that following an injection of reserpine.

Noradrenaline.—Assays of noradrenaline were attempted in all plasma samples, and very small pressor effects were seen in the pithed rat when volumes of eluate equivalent to 2 to 3 ml. of plasma were injected. Assuming that these effects were due to noradrenaline, they indicated concentrations between 0.3 and 1 μg./l. plasma, and there were no consistent differences between samples obtained before and after reserpine. These quantities were too near the threshold of the method for tests of specificity to be carried out. It was, however, ascertained that the eluate did not mask the action of added noradrenaline, so that the figures should represent the upper limits of the real noradrenaline concentration, errors due to incomplete recovery having been shown to be small (Holzbauer and Vogt, 1954).

Discussion

The rise in circulating adrenaline seen after an injection of reserpine confirms the view that the loss in amines found in adrenals and ganglia is caused by a release from the tissue and not by an inhibition of synthesis. In this respect, there is complete parallelism with the effect of reserpine on 5-HT: synthesis of 5-HT is not impaired in tissue homogenates from reserpinized rabbits (Kuntzman, Udenfriend, Tomich, Brodie, and Shore, 1956), and its urinary metabolites rise to 4 or 5 times the normal value in dogs injected with reserpine (Shore, Silver, and Brodie, 1955). The noradrenaline levels in rabbit plasma were too near the threshold of the method to yield information on any changes that might have taken place. The upper limit of 1 μg./l. is very much lower than the figures reported in man by Burger (1957), who employed a fluorimetric method using condensation with ethylene diamine for his estimations. Assuming that the method was specific enough to estimate noradrenaline only, the fall in amine concentration after sedation by reserpine in Burger's patients might be explained on the assumption that mobilization of amines by reserpine requires larger doses than those used for sedation in psychiatric work.

Except for one rabbit (Table I, No. 6) the adrenaline concentration before reserpine was higher than that of completely resting rabbits, which, according to Armin and Grant (1955), should have values below 0.01 μg./l. This is easily explained by the amount of handling to which the rabbits were subjected.

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References

Armin, J., and Grant, R. T. (1955). *J. Physiol.*, **128**, 511.
Bertler, Å., Carlsson, A., and Rosengren, E. (1956). *Naturwissenschaften*, **43**, 521.
Besendorf, H., and Pletscher, A. (1956). *Helv. Physiol. Acta*, **14**, 383.
Brodie, B. B., Pletscher, A., and Shore, P. A. (1956). *J. Pharmacol.*, **116**, 9.
Burger, M. (1957). *Arch. exp. Path. Pharmak.*, **230**, 489.
Carlsson, A., and Hillarp, N.-Å. (1956). *K. fysiogr. Sällsk. Lund Förh.*, **26**, No. 8.
Chessin, M., Dubnick, B., Kramer, E. R., and Scott, C. C. (1956). *Fed. Proc.*, **15**, 409.
Everett, G. M., Toman, J. E. P., and Smith, Jr., A. H. (1957). Ibid., **16**, 295.
Gaddum, J. H., and Lembeck, F. (1949). *Brit. J. Pharmacol.*, **4**, 401.
Holzbauer, M., and Vogt, M. (1954). Ibid., **9**, 249.
—— —— (1956). *J. Neurochem.*, **1**, 8.
de Jongh, D. K., and van Proosdij-Hartzema, E. G. (1955). *Acta Physiol. Pharmacol. Neerl.*, **4**, 175.

Kuntzman, R., Udenfriend, S., Tomich, E. G., Brodie, B., and Shore, P. A. (1956). *Fed. Proc.*, **15**, 450.
Kuschke, H. J., and Frantz, J. (1955). *Arch. exp. Path. Pharmak.*, **224**, 269.
Maxwell, R. A., Ross, S. D., Plummer, A. J., and Sigg, E. B. (1957). *J. Pharmacol.*, **119**, 69.
Muscholl, E., and Vogt, M. (1957a). *J. Physiol.*, **136**, 7P.
—— —— (1957b). Ibid., **138**, 14P.
Paasonen, M. K., and Krayer, O. (1957). *Fed. Proc.*, **16**, 326.
Shipley, R. E., and Tilden, J. H. (1947). *Proc. Soc. exp. Biol., N.Y.*, **64**, 453.
Shore, P. A., Olin, J. S., and Brodie, B. B. (1957). *Fed. Proc.*, **16**, 335.
—— Silver, S. L., and Brodie, B. B. (1955). *Science*, **122**, 284.
Vogt, M. (1952). *Brit. J. Pharmacol.*, **7**, 325.

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THE EFFECT OF DI*ISO*PROPYL FLUOROPHOSPHONATE ON THE SENSITIVITY OF RATS TO CENTRALLY ACTING DRUGS

BY

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The effect of a previous injection of an anticholinesterase, dyflos, on the sensitivity of rats to two centrally acting drugs, pentobarbitone and leptazol, has been measured. The sensitivity was determined at 12 and 35 days after birth and in full-grown animals. Though the dose of dyflos was of the order of two-thirds of the LD50, it did not affect the sensitivity of the young animals to sodium pentobarbitone and leptazol administered 20 hours later. The full-grown animals which had received dyflos in arachis oil were more sensitive to sodium pentobarbitone than litter mates which had received an injection of arachis oil only; this difference was significant at the 0.1% level. The dyflos treatment did not significantly affect the sensitivity to leptazol.

A previous paper (Elkes, Eayrs, and Todrick, 1955) described an attempt to modify the development of central nervous function in the rat by the injection of an anticholinesterase, dyflos (di*iso*propyl fluorophosphonate, DFP), regularly from the third day after birth. The results were negative. As an alternative approach to the study of the significance of cholinesterase in the central nervous system, the effect of a previous injection of DFP on the sensitivity of growing and full-grown rats to two centrally acting drugs, sodium pentobarbitone and leptazol, has been measured.

The results suggest that DFP affects the response of the full-grown animal to sodium pentobarbitone. They also indicate that the sensitivity of the rat to sodium pentobarbitone and leptazol varies with age.

MATERIALS AND METHODS

The object of the experiment was to compare the ED50 of rats previously treated with DFP with that of untreated animals. The measurement of an ED50 presents no unusual difficulties, but, since the population variance is high, it is desirable to use litter mates of the same sex at the different dose levels. This can be arranged if the number of doses employed is four. However, the comparison of the ED50 for two sets of experimental conditions rendered such a procedure impracticable. The principle adopted was therefore to balance the DFP and control groups at a given dose in respect of litter mates of the same sex, but to pay less regard to the distribution of litters and sexes between the different doses.

Another limitation in the design of the experiment arose from the use of young animals. It had previously been found that the effects of DFP were disabling for some hours (Elkes *et al.*, 1955); in the competitive conditions of a large litter, the treated animals tended to be eliminated by the mother. A modification of the standard split litter technique was therefore adopted.

Design of Experiment

Young Rats.—The experiment involved 42 litters born over a period of 18 days, and a total of 342 rats; they were of the Department of Pharmacology, Birmingham, strain. Each litter was split as close to a 3 : 3 : 4 ratio as possible, sexes being balanced during the operation; four-tenths of each of three litters were joined to form a control group, the others to form two experimental groups; they were then returned to the three mothers at random. Litters born within 48 hr. were split and grouped as they were born, and the mean age of the group was taken as the age of all the rats in the group. The larger numbers in the control groups tended to make the animals lighter than those receiving DFP; but the effect of the drug was expected to counteract this tendency at 12 days. By the thirty-fifth day the animals were feeding themselves and the two groups were expected to weigh approximately the same. In fact, the mean weight of the rats receiving DFP was 20.0 g. at 12 days compared with 18.7 g. for the controls. By 35 days the mean weights were 77.8 g. and 77.7 g. respectively.

The distribution of animals in the experiment by litter and sex may be summarized as follows: 76% of all the rats receiving DFP were paired with litter mates of the same sex which received an injection containing no DFP; a further 12% were paired with

litter mates of the opposite sex. In the test of the sensitivity of rats to leptazol the overall ratio males/females was 40/60; in the series used to test sodium pentobarbitone it was 50/50.

Owing to deaths, the population tested at 35 days was not identical with that tested at 12 days; the majority of the animals, however, were used on both occasions.

Full-grown Animals.—A fresh group of animals was used; owing to the difficulty of obtaining females, this was numerically unbalanced in respect of sex. The females (37 animals) comprised of the mothers of the litters used in the first part of the work; nothing was known of their parentage though some of them were almost certainly litter mates. The males (258 animals) were available in litters which were grouped equally into those receiving DFP and those which did not. The effect on the results of the lack of balance of the sexes was negligible, since the females were distributed equally between the treated and untreated groups.

Experimental Technique

Newborn rats are more sensitive to DFP than full-grown animals (Freedman and Himwich, 1948), but, at the same time, the depression of the brain cholinesterase activity by a single dose of the drug appears to be less (Elkes *et al.*, 1955). Rats aged between four and six weeks, on the other hand, appear to be less sensitive to DFP than full-grown animals (Elkes *et al.*, 1955). Since the aim was to give, at each age, the maximum dose of DFP compatible with survival, the following schedule was adopted.

DFP 2.2 mg./kg. in arachis oil (Boots) was injected subcutaneously on the fourth, seventh, and eleventh days after birth; the determination of the ED50 of the centrally acting drug was made on the twelfth day, 20 hr. after the last DFP injection. No further injections were given until the thirty-fourth day when 3.5 mg./kg. of DFP was injected subcutaneously; the determination of the ED50 followed 20 hr. later.

The fully grown rats at first received the same injection as the 35-day-old animals, but it was observed that the proportion of deaths was high. The dose of DFP was therefore reduced to 3.0 mg./kg. halfway through the experiment.

Animals in control groups received equivalent amounts of arachis oil. Animals were weighed prior to injection; injections were carried out using a micrometer syringe ("Agla" type).

Between the fourth and eleventh days, there were 15 deaths among the 198 animals receiving DFP, compared with 3 deaths among the 143 receiving arachis oil only. There were 12 deaths in the first 20 hr. among 165 full-grown animals injected with DFP. No deaths were recorded among 35-day-old and full-grown rats receiving arachis oil only.

Leptazol (Cardiazol, Knoll) solutions were made up freshly each day in physiological saline from the crystalline solid. On the basis of preliminary tests, dose levels of 40, 55, 70, 90, and 100 mg./kg. were selected. Sodium pentobarbitone (Veterinary Nembutal, Abbott) solutions were made up similarly; suitable doses were found to be 18, 25, 34, 45, 54, and 65 mg./kg.

Estimation of ED50 and Calculation of Results

The criterion of effectiveness of leptazol was the observation of at least one major tonic-clonic convulsion; minimal tremors or jerking movements, and pawing at the nose, which were observed in some animals, were disregarded.

The criterion of effectiveness of sodium pentobarbitone was anaesthesia, as determined by the disappearance of the response of the animal to (a) turning it over on its back, (b) pinching the tail and ear with forceps. It was found that these two tests gave parallel results. The corneal reflex was also observed. This, however, only disappeared in deep anaesthesia, and a fair proportion of the animals which failed to respond failed also to recover consciousness.

The results have been analysed statistically by the probit method as described by Finney (1952). Since "g" was too large to neglect, the more accurate form of the analysis was used. The significance of the differences between the values of the ED50 for the animals receiving DFP and for those receiving arachis oil were calculated by substituting the logarithmically transformed results in the equation

$$t = \frac{d}{\sqrt{s_1^2 + s_2^2}}$$

RESULTS

The effect of DFP on the sensitivity of rats of different ages to leptazol is shown in Table I; the results for the parallel series receiving sodium pentobarbitone are given in Table II.

TABLE I

EFFECT OF PRETREATMENT WITH DFP ON SENSITIVITY OF RATS TO LEPTAZOL

DFP injections were given as described in text.

Note on results with 35-day-old animals. The method of statistical analysis employed gives, besides the maximum likelihood estimate and fiducial limits of the ED50, a figure for the slope of the probit line "b" and its standard error. With a given number of animals, high accuracy in one estimate is attained at the expense of less accuracy in the other. The present experiments have aimed at giving accurate estimates of ED50 rather than of "b"; ten out of the twelve values obtained for "b" have shown standard errors lying between 22% and 27%. However, the standard errors of "b" in the experiments with leptazol on the 35-day-old animals were not lower, as might have been expected from the wider fiducial limits given below, but were 38% for the experimental and 35% for the control group. It must be concluded that these two sets of results possess a lower degree of accuracy than the remaining ten; no cause can be assigned to this observation.

Age (Days)	Animals Receiving DFP in Arachis Oil		Animals Receiving Arachis Oil Alone		Probability that DFP has *not* Affected Sensitivity to Leptazol
	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	
12	56	68·2 (63·0–73·7)	56	68·5 (64·3–73·0)	>0·9
35	40	75·6 (62·4–91·5)	40	66·2 (53·9–81·3)	0·3
Full-grown	53	50·4 (45·5–55·9)	54	45·2 (40·9–50·0)	0·1

TABLE II

EFFECT OF PRETREATMENT WITH DFP ON SENSITIVITY OF RATS TO SODIUM PENTOBARBITONE

DFP injections were given as described in text.

Age (Days)	Animals Receiving DFP in Arachis Oil		Animals Receiving Arachis Oil Alone		Probability that DFP has *not* Affected Sensitivity to Pento-barbitone
	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	
12	50	28·2 (24·5–32·2)	42	26·6 (23·6–29·9)	0·5
35	46	49·9 (46·5–53·6)	47	51·5 (48·3–54·8)	0·5
Full-grown	94	39·3 (35·4–43·5)	94	59·8 (55·0–65·1)	<0·001

The results do not suggest that DFP has in any way affected the sensitivity of young rats to leptazol; the fully grown animals are also not significantly affected, though, since the difference approaches the level of significance ($P=0.1$), no positive conclusion should be drawn regarding the ineffectiveness of the treatment with DFP.

The sensitivity of young rats to sodium pentobarbitone similarly shows no signs of being altered by pretreatment with DFP; the full-grown rats on the other hand are clearly more sensitive to sodium pentobarbitone 20 hr. after the DFP injection, the ED50 falling to 39 mg./kg. from the control value of 60 mg./kg.

This is significant at less than the 0.1% level.

The sensitivity of the rats to the two drugs appears to alter with age. In view of the change in the balance of the sexes between 35 days and maturity, it is necessary, before drawing conclusions, to analyse according to sex the results obtained at 12 and 35 days; leptazol seems to be equally effective against males and females at 12 days, and an indication of greater sensitivity in the male at 35 days may not be real when account is taken of the abnormal variability of this set of figures. With sodium pentobarbitone, the results do not suggest any difference between the sexes at either age.

It therefore seems permissible to compare the ED50 values at 12 and 35 days with those for the fully grown animals. The sensitivity to leptazol increases between the twelfth day after birth and full growth, the ED50 falling from 68 to 45 mg./kg. The sensitivity of the rat to sodium pentobarbitone decreases with age, the ED50 rising from 26 mg./kg. at 12 days to 52 mg./kg. at 35 days, and finally to 60 mg./kg. at full growth, that is the greater part of the change has occurred by the 35th day.

DISCUSSION

Feldberg (1950) has reviewed the evidence that acetylcholine acts as a central synaptic transmitter. While admitting the difficulty of obtaining direct evidence, such as is available for the peripheral nervous system from experiments on the perfused superior cervical ganglion of the cat (Brown, 1937), he concludes that many of the experimental results can only be satisfactorily accounted for on the assumption that acetylcholine is concerned in central synaptic transmission. Acetylcholine applied locally or injected through the vertebral or carotid artery has both stimulating and depressant central effects; anticholinesterases have similar actions, but, by comparison with acetylcholine, depression is even more pronounced and more easily obtained than stimulation (Feldberg, 1950).

The observations of Feldberg (1950) regarding the depressant effects of acetylcholine are supported by those of Richter and Crossland (1949) on the acetylcholine content of the rat brain; these workers found that this was significantly raised during anaesthesia and sleep and significantly lowered during emotional excitement, after electrical excitation, and during convulsions; they concluded that the level appeared to vary inversely with the degree of activity of the brain.

More recently, Sherwood and Feldberg (1954) have shown that the effects of intraventricular injections of eserine and DFP in the cat take place in three stages. The first is characterized by the appearance of itching or irritation, the second by changes in gait, stance and posture, increased tone of limb muscles and tremor, and the third by an alteration of awareness including the development of stupor resembling catatonia.

These results all point to the importance of the depressive component of the action of acetylcholine on the central nervous system. It appears to us that the positive result which we have observed, namely the increase in sensitivity of the mature rat to a central depressant drug caused by a heavy dose of DFP, is not inconsistent with the results quoted above. It is perhaps of interest that the only noticeable symptom observed 20 hr. after the injection of DFP was a marked "retardation" characterized by the animal remaining motionless in a crouching position, but even this was not observed in many of the animals.

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References

Brown, G. L. (1937). *Physiol. Rev.*, **17**, 485.

Elkes, J., Eayrs, J. T., and Todrick, A. (1955). *Proc. 1st Int. Neurochem. Symp., Biochemistry of the Developing Nervous System*, p. 499. New York: Academic Press.

Feldberg, W. (1950). *Brit. med. Bull.*, **6**, 312.

Finney, D. J. (1952). *Probit Analysis*, 2nd ed., p. 236. Cambridge: University Press.

Freedman, A. M., and Himwich, H. E. (1948). *Amer. J. Physiol.*, **153**, 121.

Richter, D., and Crossland, J. (1949). Ibid., **159**, 247.

Sherwood, S. L., and Feldberg, W. (1954). *J. Phy siol.* **125**, 488.

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337C54. *See* 2 : 5-Di(*cyclo*hexylaminomethyl)naphthalene-1 : 6-diol
883 F. *See* Prosympal

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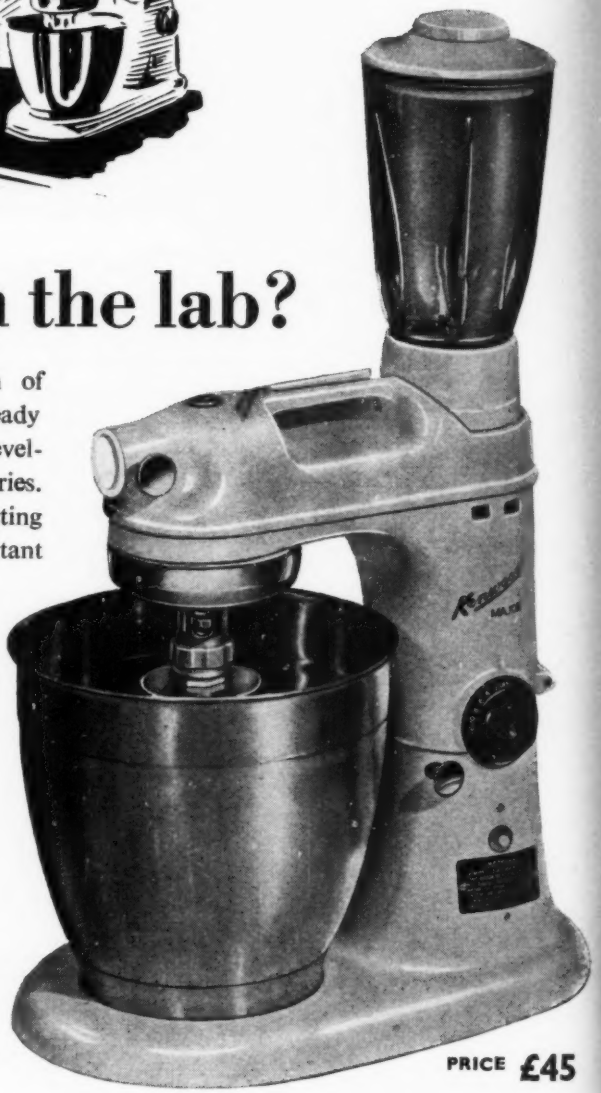

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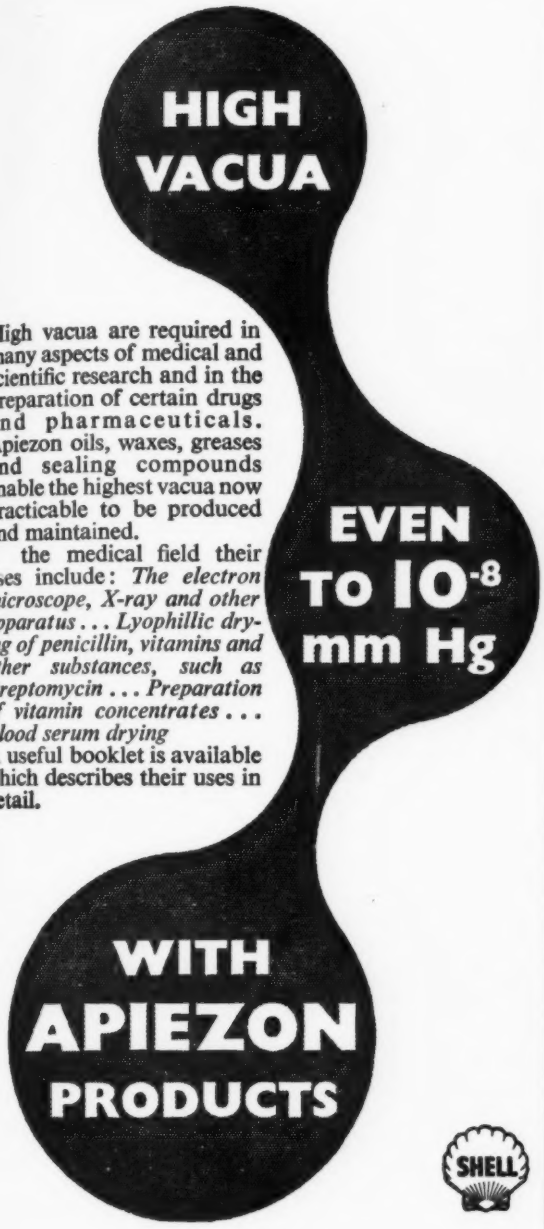

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